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# POLYPHENOL CONTENT AND DIFFERENTIAL EXPRESSION OF FLAVONOID BIOSYNTHETIC PATHWAY GENES OF *FRAGARIA* SPP. WITH WHITE FRUIT

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DISSERTATION

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A Dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By

Sutapa Roy

Director: Dr. Douglas D. Archbold, Professor, Department of Horticulture

Lexington, Kentucky

2016

ABSTRACT OF DISSERTATION

# POLYPHENOL CONTENT AND DIFFERENTIAL EXPRESSION OF FLAVONOID BIOSYNTHETIC PATHWAY GENES OF *FRAGARIA* SPP. WITH WHITE FRUIT

Strawberries are a rich source of polyphenols which contribute to berry color and plant disease resistance, and have been shown to lower the risk of many chronic when consumed. While a considerable body of work exists on the polyphenolic composition of commercial strawberry (*Fragaria x ananassa* Duch.), less information is available concerning polyphenols in *Fragaria vesca*, or Alpine strawberry, considered a model system for the Rosaceae family of crop species. The study of natural and genetically-engineered *F. vesca* mutants with white fruit can provide unique insight into regulation of metabolic flux through the complex branched phenylpropanoid/flavonoid biosynthetic pathway. Thus, the identity and quantity of major phenolic-derived anthocyanins, flavonols, flavan-3-ols, hydroxycinnamic acids, and ellagic acid (EA)-derived compounds, of red-fruited versus white-fruited genotypes of *F. vesca* and *F. x ananassa* were compared by high performance liquid chromatography-mass spectrometry. Due to the unknown origin of all but one white-fruited mutant of *F. vesca*, it was assumed that each resulted from independent mutation events and would exhibit different flavonoid profiles. A total of 27 phenolic-derived compounds were identified. The white genotypes of both species had very low anthocyanin levels. Total content of free EA and its conjugated forms were generally higher in white than in red *F. vesca,* but were the opposite in *F. x ananassa,* more in red than in white berries. Differences in content of individual flavonoids and in group totals among the white *F. vesca* genotypes suggested that they may represent different mutations affecting flavonoid production. Polyphenol profiles of a red and a white cultivar of *F. vesca* during four fruit developmental stages were determined along with transcriptional analyses of key structural and regulatory genes of the phenylpropanoid/ flavonoid biosynthesis. The final concentration of polyphenolic groups in red versus white *F. vesca* was due to the differential expression patterns of key pathway genes, especially dihydroflavonol-4-reductase, anthocyanidin synthase, and UDP-glucose-flavonoid-3-*O*-glucosyltransferase. The efficacy of phenolic compounds were evaluated in an *in vitro* study for inhibiting growth of *Colletotrichum* spp. associated with anthracnose fruit rot of strawberry. Only *trans*-cinnamic, *p*-coumaric, and ferulic acid inhibited isolates of the pathogen.

KEYWORDS: polyphenol, strawberry, flavonoid, anthocyanin, proanthocyanidin

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Student Signature

03-11-2016

Date

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## Chapter 1: Introduction

Phenolic-derived compounds, including flavonoids and ellagitannins, are the most widely distributed and chemically diverse group of secondary metabolites synthesized by plants (Crozier *et al*., 2006). These compounds often accumulate in relatively high amounts in plant tissues, and increase the capacity of plants to survive (Macheix *et al*., 2005). They are reportedly involved in growth and reproduction, tissue pigmentation, defense against pathogens and insect pests, attraction of pollinators and seed-dispersing animals, protection against UV light, and signaling between plants and other organisms (Lattanzio *et al*., 2006; Treutter, 2006). In addition to their roles in plants, polyphenols also provide sensory and health-beneficial attributes to fruits and vegetables in the human diet, influencing flavor, color and antioxidant activity (Craig, 1999; Kumar *et al*., 2012). Phenolic acids, flavonoids, and tannins have been associated with reductions in some health problems (Steinmetz and Potter, 1996; Riboli and Norat, 2003) such as cardiovascular disease and cancer (Yao *et al*., 2004; Yang *et al*., 2007; Jagota *et al*., 2007; Jochmann *et al*., 2008; Lin *et al*., 2009; Lima *et al*., 2014). Natural phenolic compounds have been shown to provide atherosclerosis protection, radio-protective action, and antimicrobial, antioxidative and anti-inflammatory activities (Yao *et al*., 2004; Hogan *et al*., 2010; Shen *et al*., 2011). For example, crude strawberry (*Fragaria x ananassa* Duch.) extracts and purified compounds inhibited the growth of human oral, colon, and prostate cancer cells in a dose-dependent manner with various degrees of potency (Zhang *et al*., 2008).

The effectiveness of polyphenols is primarily due to their activities as reducing agents, hydrogen donors, and singlet oxygen quenchers (Rice-Evans *et al*., 1997). Their antioxidant properties as radical scavengers and metal chelators are dependent on their structure, which may vary considerably with the degree of hydroxylation, substitution, conjugation and polymerization (Kelly *et al*., 2002). These types of results have led researchers to become more interested in understanding and exploiting polyphenols as bioactive compounds in human health. For these reasons, the characterization and quantification of these polyphenolic compounds in different plant tissues have grown in importance. Furthermore, research on the shikimate and phenylpropanoid/flavonoid biosynthetic pathways that produce these compounds, on regulation of the genes involved, and on bioengineering changes and/or improvements in polyphenol content and quantity has increased (Carbone *et al*., 2009; Lin-Wang *et al*., 2014).

Strawberries belong to the genus *Fragaria* which varies in ploidy levels and plant morphology, and which is comprised of a number of species. An abundance of studies have focused on determining phenolic compound identity and concentration in fruit of cultivars of the octoploid commercial species, *F. x ananassa* (Seeram *et al*., 2006; Aaby *et al*., 2007; 2012; Buendia *et al*., 2010; Kelebek *et al*., 2010). However, similar studies of the diploid strawberry *F. vesca* are few. The diploid *F. vesca* has recently become a model species for *F. x ananassa*, and even for the members of the Rosaceae family, which includes apple, peach, cherry, plum, and rose. Due to the importance of polyphenolic compounds to these crops, there has been increased interest in both characterizing the polyphenolic compounds and understanding the influence of specific components of the biosynthetic pathway on their production. The long-term goals are to breed superior cultivars by traditional methods combined with molecular techniques (Hancock et al., 2008), and to directly engineer improvements by targeting key factors for change (Lin-Wang *et al*., 2014).

There are several white-fruited mutants of *F. vesca* available, most of which have not been studied. The origin of most is not known, so they may have been the result of independent mutations, and thus differ in their polyphenolic profiles. Therefore, the objectives of the present research were several-fold. The identity and content of target polyphenolic compounds of ripe fruit of several white *F. vesca* mutants were studied, for comparison among them as well as to red *F. vesca* fruit, considered wild-type, and to red and white *F. x ananassa*. This comparison provided an opportuntity to determine how alterations in the biosynthetic pathway alter polyphenolic compound profiles, with the hypothesis that each white genotype was due to a unique mutation and would result in differing polyphenolic profiles. In addition, the developmental patterns of accumulation of the major phenolic compounds with comprehensive parallel characterization of structural and regulatory genes in the phenylpropanoid/flavonoid biosynthetic pathway in a red versus a white-fruited *F. vesca* were compared to determine how the final ripe fruit polyphenol profiles evolve and differ. Finally, because polyphenols have been suggested as components of plant defense against pathogens, and significant differences in polyphenol content and/or profiles of red versus white fruit may exist, the *in vitro* effect of phenolic acids on growth of different *Colletotrichum* spp., the pathogen causing anthracnose in strawberry, was examined.

# Chapter 2: Literature Review

## 2.1 *Fragaria vesca* as a model species for strawberry and other Rosaceae

The genus *Fragaria* is a member of the Rosaceae family subfamily Rosoideae. Closely-related genera include *Duchesnea*, the mock strawberry, and *Potentilla*, the cinquefoils. Among the 20 named species of *Fragaria*, four levels of ploidy can be found; there are 12 diploids, 2 tetraploids, 1 pentaploid, 1 hexaploid, and 4 octoploids (Hancock *et al.*, 2008). Diploids (2n=14) include the most common wild species, *F. vesca* Duch. (Staudt, 1989). The octoploid commercial strawberry *F. x ananassa* is the most economically important and the most cultivated strawberry worldwide, and is a hybrid of *F. chiloensis* and *F. virginiana* (Staudt, 1962)

*F. vesca,* the Alpine or woodland strawberry, is a self-pollinating diploid with a relatively small genome size (164 Mbp/C) which is comparable to that of the model plant species *Arabidopsis thaliana* (Akiyama *et al*., 2001). Because of its morphological diversity and interfertility (Hancock *et al.,* 2008), the full *F. vesca* genome was recently characterized (Shulaev *et al*., 2011). The small plant size, transformability with *Agrobacterium*, small genome size, short intergenerational period in comparison to the tree fruit tree crops in the Rosaceae, available genome sequence of *F. vesca* var. Hawaii 4, high degree of synteny between *F. vesca* and the octoploid commercial varieties of *F* x *ananassa* (Tennessen *et al*., 2009) and other Rosaceae family species such as peach (*Prunus persica*) and apple (*Malus* x *domestica*) (Vilanova *et al*., 2008; Rousseau-Gueutin *et al*., 2008; Jung *et al*., 2012), and existing inbred lines have led many to consider *F. vesca* a useful model system for *Fragaria* genus and possibly other Rosaceae species. The USDA *Fragaria* germplasm repository at Corvallis, Oregon, contains common red-fruited types as well as white mutants of *F. vesca*, *F. chiloensis,* and commercial *F. x ananassa* strawberry cultivars.

## 2.2 Structure and biosynthesis of simple and complex phenolic compounds in *Fragaria* spp.

### 2.2.1 Phenolic compounds in *Fragaria* spp.

The content of polyphenols in *Fragaria* spp., primarily flavonoids and ellagitannins, is highly dependent on cultivar, site and environmental conditions during production, postharvest practices, and processing conditions (Harnly *et al*., 2006; Carbone *et al.*, 2009).

Many studies have reported the identity and/or concentration of polyphenols in varieties of *F. x ananassa.* However, there are far fewer studies on polyphenol biosynthesis in *F. x ananassa* during fruit development, or on the polyphenol biosynthesis and composition of other *Fragaria* spp.

#### 2.2.1.1 Phenolic acids

Phenolic acids may exist in free or bound forms in plants, where they may be linked to other compounds through ester, ether, or acetal bonds (Robbins, 2003; Zadernowski *et al.,* 2009; Ignat *et al*., 2011). Phenolic acids often differ from one another by the number of hydroxy and methoxy groups on their phenyl unit (Macheix *et al.,* 1990). They can be subdivided into hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids have a general structure of a phenyl ring with a single carbon side chain (C6–C1) and include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids. Hydroxycinnamic acids have a three-carbon side chain attached to the general aromatic ring (C6–C3). Four of the most common hydroxycinnamic acids are caffeic, ferulic, *p*-coumaric and sinapic acids (Bravo, 1998). These monophenolic carboxylic acids are often found as esters of quinic, shikimic and tartaric acid, as well as sugar derivatives (Schuster and Herrmann, 1985; Macheix *et al.* 1990; Shahidi and Naczk, 1995). Caffeic acid (3, 4-dihydroxycinnamic acid) may be conjugated with quinic acid to form chlorogenic acid.

The most common phenolic acids in *F. x ananassa* cultivars are hydroxycinnamic acids, such as *p*-coumaric acid, caffeic acid, and ferulic acid, and their derivatives. *p*-Coumaroyl hexose, ferulic acid hexose derivatives and caffeoyl hexose have all been identified in *F. x ananassa* (Seeram *et al*., 2006; Aaby *et al*., 2007, 2012; Buendia *et al*., 2010; Kelebek *et al*., 2010). Simirgiotis *et al*. (2010) detected only conjugated forms of *p*-coumaric acid in both red- and white-fruited *F. chiloensis*. Free *p*-coumaric acid, *p*-coumaroyl glucose, chlorogenic acid, caffeic acid, ferulic acid, and ferulic acid glucose were reported in red *F. vesca* (Del Bubba *et al*., 2012; Sun *et al*., 2014). Aaby *et al.* (2012) and Medina-Puche *et al*. (2013) compared *F. x ananassa* red cultivars during fruit development and found a higher accumulation of *p*-coumaroyl hexose in the red ripe stage than at the turning stage. The total hydroxycinnamic acid content of *F. x ananassa* cultivars has been reported to range from 0.8 to 6.7 mg/100 g FW (Buendia *et al.*, 2010; Kelebek *et al*., 2011). A comparative analysis by Munoz *et al*. (2011) showed a range of 0.3 to 0.9 mg/100 g FW phenolic acid

derivatives in five different *Fragaria* spp. *F. chiloensis* had the greatest amount, followed by a *F. x ananassa* cultivar, and the least in a *F. vesca* cultivar.

#### 2.2.1.2 Flavonoids

Flavonoids have a benzo-γ-pyrone structure, consisting of fifteen carbon atoms arranged in a $C_6$-$C_3$-$C_6$ configuration with two benzene rings (A and B) joined via a heterocyclic pyran, or ring C (Jaganath and Crozier, 2010). Flavonoids from higher plants are divided into different subclasses including chalcones, flavones, flavonols, flavans, anthocyanins, and proanthocyanidins (or condensed tannins) (Winkel-Shirley, 2001). Flavonoids are often classified according to their cyclization, degree of unsaturation, and oxidation of the C ring, whereas substitutions on rings A and B are responsible for differences among compounds within the same class of flavonoids (Kumar and Pandey, 2013)

The basic flavonoid structure is an aglycone, which may then be modified by hydroxylation, methylation, and glycosylation. Flavonoids are most commonly found as glycosides where one or more hydroxyl groups are attached to a sugar via an acid-labile hemiacetal O-C bond (*O*-glycosides), or are bound to the flavonoid aglycone via an acid-resistant C-C bond (C-glycosides) (Rak *et al*., 2010). Glycosylation makes the flavonoids less reactive and more water-soluble which prevents cytoplasmic damage and allows storage of the flavonoids in the cell vacuole. Flavonoids can be hydroxylated at the $C_2$, $C_3$, $C_5$, $C_7$, $C_3$', $C_4$' and $C_5$' positions, and classes differ from each other with respect to their glycosylation positions: flavonones, flavones and isoflavones are glycosylated at $C_7$, flavonols and flavanols at $C_3$ and $C_7$, while anthocyanidins have glucose moieties at the $C_3$ and $C_5$ hydroxyl groups (Cuyckens and Claeys, 2004). The sugars involved can be glucose, galactose, rhamnose, fructose or arabinose. Acylated and methylated glycosides are also known to occur in plants (Kumar and Pandey, 2013).

Quercetin and kaempferol are the major flavonol aglycones in *Fragaria* spp. Among *F. x ananassa* cultivars, quercetin-3-glucoside, quercetin-3-glucuronide, quercetin malonylglucoside, and quercetin-3-pentoside have been reported (Aaby *et al*., 2007; 2012; Buendia *et al*., 2010; Kajdžanoska *et al*., 2010; Kelebek *et al*., 2011). The kaempferol conjugates kaempferol-3-glucoside, kaempferol-3-coumaroyl glucoside, kaempferol-3-malonyl glucoside, and kaempferol acetyl glucoside have been reported in *F. x ananassa*

(Seeram *et al*., 2006; Aaby *et al*., 2007, 2012; Buendia *et al*., 2010; Kajdžanoska *et al*., 2010; Kelebek *et al*., 2011). Simirgiotis *et al*. (2009) reported the presence of quercetin pentoside, quercetin hexoside, quercetin glucuronide, kaempferol glucuronide, and kaempferol coumaroyl hexoside in red and white forms of *F. chiloensis*. Kaempferol hexoside, kaempferol acetyl hexoside, kaempferol coumaroyl hexoside, quercetin glucoside, quercetin glucoronide and quercetin acetyl hexoside were reported in red and white cultivars of *F. vesca* (Del Bubba *et al*., 2012; Sun *et al*., 2013). Total flavonol concentration ranged from 0.5 to 3.4 mg/100 g FW (Buendia *et al.*, 2010; Kelebek *et al*., 2011; Aaby *et al*., 2012) among cultivars of *F. x ananassa.* Simirgiotis *et al.* (2009) reported quercetin content as 0.8 mg/100 g FW and 0.5 mg/100 g FW, and kaempferol content as 1.08 mg/100 g FW and 0.9 mg/100 g FW, for the red and white *F. chiloensis*, respectively. In contrast, Munoz *et al.* (2011) reported a lower content of total flavonols in all three *Fragaria* spp. (close to 0.004 mg/100g FW) in comparison to the other studies. Kaempferol-3-*O*-glucoside was the most abundant flavonol in *F. x ananassa* and *F. vesca,* followed by quercetin-3-*O*-glucoside in *F. vesca* and quercetin-3-*O*-glucuronide in *F. x ananassa* and *F. chiloensis.*

#### 2.2.1.3 Anthocyanins

Anthocyanins, end products of the flavonoid biosynthesis pathway, are water-soluble pigments that provide plant tissues of many species a diverse range of red, blue and purple colors (Robards and Antolovich, 1997). Anthocyanins consist of an anthocyanidin aglycone, which is bound to one or more sugar moieties, typically attached at the 3-position of the C-ring, but additional sugar residues at the 5 and 7 positions are also possible (Robards and Antolovich, 1997; Jaganath and Crozier, 2010). Glucose, arabinose, and galactose are the most common sugar moieties. Anthocyanidins are hydroxylated and methoxylated derivatives of phenyl-2-benzopyrylium and exist in cationic form in acidic medium with numerous derivatives displaying stability (Siddiq *et al*., 1994). There are about 17 anthocyanidins found in nature, but 6 are the most widespread and contribute to the pigmentation of fruits including *Fragaria* spp. These 6 anthocyanidins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin. Cyanidin is the most common one found in plants, followed by delphinidin and pelargonidin. Delphinidin, with its derivatives petunidin and malvidin, is responsible for a dark-bluish color, whereas pelargonidin and cyanidin provide dark-reddish colors. The variation among anthocyanins is mainly due to the number and position of hydroxyl and methoxy groups on the

anthocyanidin aglycone, the types, numbers, and position of sugars on the aglycone, and the acylation extent of the sugar (Jaganath and Crozier, 2010).

There are three major anthocyanidins found in *Fragaria* spp.: pelargonidin, cyanidin and peonidin. A derivative of pelargonidin, pelargonidin-3-glucoside, was the most abundant anthocyanin in many red cultivars of *F. x ananassa,* contributing 60-95% of total anthocyanin content (Aaby *et al*., 2007, 2012; Lopes-da-Silva *et al*., 2007; Buendia *et al*., 2010). Pelargonidin malonylglucoside was the second most abundant anthocyanin present in *F. x ananassa* cultivars followed by low levels of pelargonidin malonylrhamnoside, pelargonidin rutinoside and pelargonidin acetylglucoside (Lopes-da-Silva *et al*., 2002; Aaby *et al*. 2007, 2012; Buendia *et al*., 2010; Cerezo *et al*., 2010; Kelebek *et al*., 2010; Kajdžanoska *et al*., 2010). Three cyanidin derivatives, cyanidin-3-glucoside, cyanidin malonylglucoside and cyanidin rutinoside, were the predominant anthocyanins in a few *F. x ananassa* varieties (Seeram *et al*., 2006; Aaby *et al*., 2007, 2012; Simirgiotis *et al*., 2009; Cerezo *et al*., 2010; Buendia *et al*., 2010, Kelebek *et al*., 2010;). The presence of peonidin-3-glucoside was first detected by Cerezo *et al*. (2010) in *F. x ananassa*. Simirgiotis *et al*. (2009) reported the presence of peonidin derivatives in *F. chiloensis*. Cyanidin and pelargonidin derivatives were found in white forms of *F. chiloensis*, though in very low amounts. Peonidin-3-glucoside, peonidin malonylglucoside, cyanidin-3-glucoside, and cyanidin malonylglucoside were observed in red cultivars of *F. vesca* (Del Bubba *et al*., 2012; Sun *et al*., 2013). Accumulation of anthocyanins occurred during the later stages of ripening in all red cultivars of the three *Fragaria* spp. (Carbone *et al*., 2009; Salvatierra *et al*., 2010; Xu *et al*., 2014b). Total anthocyanin concentration of 15 cultivars of *F. x ananassa* ranged from 23.5 to 47.4 mg/100 g FW (Buendia *et al.,* 2010), and of 27 cultivars of *F. x ananassa* ranged from 8.5 to 65.9 mg/100 g FW (Aaby *et al*., 2012). The red and white forms of *F. chiloensis* had 22.5 mg and 2.2 mg/100 FW of total anthocyanins, respectively (Simirgiotis *et al*., 2009). The concentration of pelargonidin-3-glucoside was higher than cyanidin-3-glucoside in a *F. x ananassa* and a *F. chiloensis* cultivar, but the levels for both were similar in a red *F. vesca* (Munoz *et al.*, 2011). However, the total anthocyanin content was very low in the three species in this paper, ranging from 1 to 4 mg/100 g FW. In some recent studies of red *F. vesca* genotypes, the range of total anthocyanin content was 25 to 51 mg/100 g FW in 15 genotypes (Yildiz *et al*., 2014), while Najda *et al*. (2014) reported 80 to 90 mg/100 g FW in two genotypes.

### 2.2.1.4 Proanthocyanidins and ellagitannins

#### 2.2.1.4.1 Proanthocyanidins

Tannins are a major group of polyphenols, which can be classified into three groups; the proanthocyanidins (condensed tannins), the gallo- and ellagitannins (hydrolysable tannins), and the phlorotannins. Proanthocyanidins or condensed tannins are oligomeric and monomeric end products, which are derived from condensation reactions of flavan-3-ol (or flavanol) monomeric units. The position, region, and stereochemical variations of the flavanol interlinkages, degree of polymerization, changes in the phenolic hydroxylation pattern, and modifications such as esterification of the 3-hydroxyl group of the C-ring, determine the structure of this family of flavonoids (Dixon *et al.*, 2005; Quideau *et al.*, 2011). The stereochemical difference of the monomeric units consists of 2, 3-*cis* forms in (-) epicatechin, and 2, 3-*trans* forms in (+) catechin. Proanthocyanidins are oligomers of catechin and epicatechin with gallic acid esters. Generally, proanthocyanidins are divided into A and B types, depending on the single or double linkages connecting two flavan-3-ol units. The linkages in B-types are between the $C_4$ of the upper unit and the $C_8$ of the lower unit, although it can also be between the $C_4$ of the upper unit and the $C_6$ of the lower unit. A-Type proanthocyanidin linkages may occur between both the $C_2$ and $C_4$ of the upper unit, with an additional ether bond at an oxygen at $C_7$ and $C_6$ or $C_8$ of the lower unit (Ferreira *et al.*, 2003; Dixon *et al.*, 2005; Quideau *et al.*, 2011). A-Type proanthocyanidins are not present in strawberry. Proanthocyanidins formed with similar types of oligomers (two 3'4' hydroxyl groups) are termed procyanidins, whereas proanthocyanidins containing mixed oligomers with one 4'-hydroxyl are termed propelargonidin, and those with a 3'4'5'-tri-hydroxyl pattern are known as prodelphinidins. Prodelphinidins have not been found in plants from the Rosaceae family, including *Fragaria* spp.

Many studies of *F. x ananassa* have shown a range in compound size, from simple monomeric catechin to proanthocyanidins with varying and high degrees of polymerization (Aaby *et al.*, 2007, 2012; Buendia *et al.*, 2010). The most abundant proanthocyanidins are dimers of proanthocyanidins identified as proanthocyanidin B1 and proanthocyanidin B3, or simply as proanthocyanidin dimers (Aaby *et al.*, 2007, 2012; Kajdžanoska *et al.*, 2010; Josuttis *et al.*, 2013). Other reported polymers are proanthocyanidin trimers, tetramers, and

pentamers (Aaby *et al*., 2007, 2012; Kajdžanoska *et al*., 2010; Simirgiotis *et al.*, 2009; Josuttis *et al*., 2013). Simirgiotis *et al*. (2009) found only proanthocyanidin tetramers in the red-fruited *F. chiloensis*. In *F. vesca*, monomeric catechin and epicatechin were reported along with B-type proanthocyanidin, B-type epicatechin trimers, and proanthocyanidin trimers and tetramers (Del Bubba *et al*., 2012; Sun *et al*., 2013). The general pattern of proanthocyanidin content during fruit development was a decline from the earlier stages to the final stages of fruit development in *F. x ananassa* (Fait *et al*., 2008; Carbone *et al.*, 2009). Total proanthocyanidin concentration of 15 cultivars of *F. x ananassa* was 54 to 163 mg/100 g FW (Buendia *et al*., 2010), but values for *F. vesca* and *F. chiloensis* have been reported to be a little more than 0.001 mg/100 g FW (Munoz *et al.,* 2011).

**2.2.1.4.2 Ellagitannins and ellagic acid conjugates**

Hydrolysable tannins have a typical central sugar core (most commonly β-D-glucose) to which gallic acid-derived motifs are esterified (Quideau *et al.*, 2011). Multiple galloyl units forming β-D-glucopyranose β-pentagalloyl glucose is considered as the precursor of two types of hydrolysable tannins, gallotannins and ellagitannins. Gallotannins result from galloylation of pentagalloyl glucose. Phenolic oxidative coupling of two adjacent glucopyranoses can generate hexahydroxydiphenoyl (HHDP) units, characteristic of ellagitannins. The diversity of structures of ellagitannins is because of the many region-isomeric HHDP connections on the glucopyranose scaffold (Feldman *et al*., 2003; Quideau *et al.*, 2011). Hydrolysis of ellagitannin releases HHDP units forming free ellagic acid (EA).

Ellagitannins and ellagic acid conjugates are present at relatively high concentrations in *Fragaria* spp. fruit. There is a developing yet incomplete knowledge regarding qualitative and quantitative analyses of ellagitannins in *Fragaria* spp. The presence of ellagitannin in *F. x ananassa* includes simple galloyl bis HHDP glucose, HHDP galloyl glucose, digalloyl diHHDP glucose and complex dimers of galloyl bis HHDP glucose, including argimonin, sanguiin H6 and sanguiin H10 (Aaby *et al*., 2007, 2012; Buendia *et al*., 2010; Kajdžanoska *et al*., 2010; Gasperotti *et al.*, 2013). Polyphenolic profiling of red cultivars of *F. vesca* showed an abundance of bis HHDP hexose, HHDP galloyl hexose, and casurictin-like ellagitannin which is also known as galloyl bis HHDP glucose (Del Bubba *et al*., 2012; Sun *et al*., 2013). In addition to a higher amount of free EA, several EA derivatives have been reported for red *F. x ananassa* cultivars, including EA pentoside, EA

deoxyhexoside, methyl EA deoxyhexoside, EA rhamnoside, EA hexoside and methyl EA pentoside (Seeram *et al*., 2006; Aaby *et al*., 2007, 2012; Buendia *et al*., 2010; Kajdžanoska *et al*., 2010; Gasperotti *et al*., 2013; Sun *et al*., 2013). In *F. vesca,* EA pentoside, EA rhamnoside, methyl EA hexoside, dimethyl EA pentoside, and EA deoxyhexoside were reported (Del Bubba *et al*., 2012; Sun *et al*., 2013; Gasperotti *et al.*, 2013).

Ellagitannin and EA derivatives were at higher concentrations in the early stages of fruit development for commercial strawberry with a decline in content in the later stages (Fait *et al*., 2008; Carbone *et al*., 2009). The ellagitannin content of *F. x ananassa* and red and white fruits of *F. vesca* at different stages of fruit development showed variation in the presence and content of ellagitannins and ellagic acid derivatives among genotypes (Gasperotti *et al.*, 2013). Red and white *F. vesca* fruits showed a less pronounced drop in ellagitannin concentration from earlier to later stages than *F. x ananassa,* but *F. x ananassa* had higher ellagitannin content in the early fruit development stages (Gasperotti *et al*., 2013). Several studies have reported total ellagic acid and ellagic acid derivative content of cultivars of *F. x ananassa* that ranged from 0.1 to 0.5 mg/100 g FW (Kosar *et al*., 2004) and 0.1 to 0.8 mg/100 g FW (Aaby *et al*., 2012) for ellagic acid, and 0.9 to 1.8 mg/100 g FW (Buendia *et al*., 2010) and 0.1 to 1.8 mg/100 g FW (Aaby *et al*., 2012) for total ellagic acid derivatives. Gasperotti *et al.* (2013) reported total EA derivatives and ellagitannin content in 6 cultivars of *F. x ananassa* as 26.2 to 36.9 mg/100 g FW. Simirgiotis *et al.* (2010) only reported the values of free ellagic acid for red and white forms of *F. chiloensis* as 1.8 mg and 5.9 mg/100 g FW, respectively. In *F. vesca,* higher amounts of total EA derivatives and ellagitannins were found for both red and white forms. Gasperotti *et al*. (2013) reported 85.9 mg/100 g FW for ripe fruits of red *F. vesca* and 71.9 mg/100 g FW for the white fruit.

#### 2.2.1.5 Pholorotannins

Phlorotannins are oligomers of phloroglucinols, but they have not been reported in the Rosaceae family including *Fragaria* spp., only in red-brown algae (Sailler *et al*., 1999).

### 2.3 Biosynthetic pathways of phenolic and flavonoid compounds

Biosynthesis of phenolics and flavonoids involves the contributions of the shikimate, phenylpropanoid/flavonoid, and acetate-malonate biosynthetic pathways. Each pathway consists of several steps, which are common to the synthesis of a number of different

secondary metabolites, and branch steps, which are more specific for individual metabolites.

### 2.3.1 Shikimate pathway

The shikimate pathway converts two metabolites, phosphoenol pyruvate (PEP) of the glycolytic pathway and erythrose-4-phosphate (E4P) of the non-oxidative branch of the pentose phosphate pathway, into chorismate (Fig. 2.1) (Muir *et al*., 2011). Chorismate is responsible for the production of three aromatic amino acids, tyrosine, tryptophan and phenylalanine. Phenylalanine is an essential precursor for many secondary metabolites, particularly phenolic acids and flavonoids. Another important branch route of the shikimate pathway leads to the biosynthesis of gallic acid, which is the primary building block of all hydrolysable tannins, the gallotannins and ellagitannins. At one point in the shikimate pathway, 3-dehydroshikimic acid can either be reduced to shikimic acid by shikimate dehydrogenase, or produce 3,5-dehydroshikimic acid which, with subsequent enolization, produces gallic acid (Haslam and Cai, 1994; Muir *et al.*, 2011). Gallic acid or gallic acid-derived motifs are esterified with a glucose unit by 1-O-galloyl transferase (Li *et al*., 1999; Michel *et al.*, 2003) to produce different glucosyl gallates. β-Glucogallin is the simplest glucosyl gallate, which serves as a galloyl unit donor in the biosynthesis of β-D-glucopyranose-β-pentagalloyl glucose (Quideau *et al.,* 2011). Gallotannins are formed by gallolyation of these pentagalloyl glucoses and may contain six or more galloyl units. Ellagitannin is the oligomer or polymer of HHDP units. HHDP is the product of intra- and intermolecular oxidation of pentagalloyl glucose. One or more galloyl groups are linked to the HHDP units via C-C biaryl and C-O diaryl ether bonds (Okuda *et al*., 2009). The hydrolysable tannin pathway is very complex and has not been well-characterized. The presence and oligomerization of ellagitannins differs among species (Okuda *et al.*, 2009).

### 2.3.2 Phenylpropanoid/flavonoid biosynthetic pathway

The formation of flavonoids, isoflavonoids and lignins is initiated by the phenylpropanoid pathway followed by the flavonoid biosynthetic pathway (Bohm, 1998). Phenylalanine is the initial precursor for this combined biosynthetic pathway, derived from the shikimate pathway discussed above (Fig. 2.1), which is converted to cinnamic acid by the activity of the enzyme phenylalanine ammonia lyase (PAL) (Fig. 2.2). Cinnamic acid 4-hydroxylase forms *p*-coumaric acid, and when a second hydroxyl group is introduced onto

*p*-coumaric acid in the presence of mono-oxygenase by *p*-coumaroyl shikimate/quinate-3'-hydroxylase (C3'H), caffeic acid is produced. Caffeic acid can further be methylated by caffeic acid/5-hydroxyferulic acid–*O*-methyltransferase (COMT) to form ferulic acid, which may be converted to 5-hydroxyferulic acid (Nair *et al*., 2004). *p*-Coumaric acid may also be converted to 4-coumaroyl CoA with the help of hydroxycinnamate CoA ligase (C4L).

The flavonoid biosynthetic pathway begins with the formation of chalcone from the condensation of one molecule of 4-coumaroyl–CoA and three molecules of malonyl CoA by a type III polyketide-synthase known as chalcone synthase (CHS). Malonyl CoA comes from the acetate/malonate pathway. Next, chalcone is isomerized to the (2*S*)-flavanone naringenin by chalcone isomerase (CHI). Naringenin is at a branch point and can give rise to different final products including flavanones, flavonols, flavanols, proanthocyanidins and anthocyanins, depending on the species. Naringenin can go through oxidative rearrangement to form phytoalexins, a double bond can be introduced leading to the formation of flavones, or hydroxylation can take place, forming dihydroflavonols. The third reaction producing dihydroflavonols is the primary one observed among these alternatives in fruit including strawberry. The stereospecific 3β hydroxylation of (2*S*)-flavanones to dihydrokaempferol (DHK) is done by flavanone-3-hydroxylase (F3H), a member of the 2-oxo-glutarate family. Dihydrokaempferol acts as a precursor for flavonols, anthocyanidins, flavan-3-ols and proanthocyanidins. β-Ring hydroxylation is an important event which determines the color of fruit and the formation of flavonols and anthocyanidins with different biochemical and antioxidant properties. The 4' hydroxyl group of the β ring of naringenin is incorporated from 4-coumaroyl CoA during the condensation reaction with malonyl CoA. Hydroxyl group introduction at the 3' or both the 3' and 5' positions of the β ring of dihydrokaempferol to create dihydroquercetin and/or dihydromyrcetin results from the activity of cytochrome P450 monooxygenase, flavanone-3'-hydroxylase (F3'H) and/or flavanone 3'5' hydroxylase (F3'5'H), respectively. In members of the Rosaceae family such as apple (*Malus* spp.), rose (*Rosa* spp.) and strawberry (*Fragaria* spp.), no 3'4'5' hydroxylated flavonoids are present due to absence of F3'5'H enzyme (Elomaa and Holton, 1994; Bogs *et al*., 2006; Castellarin *et al*., 2006).

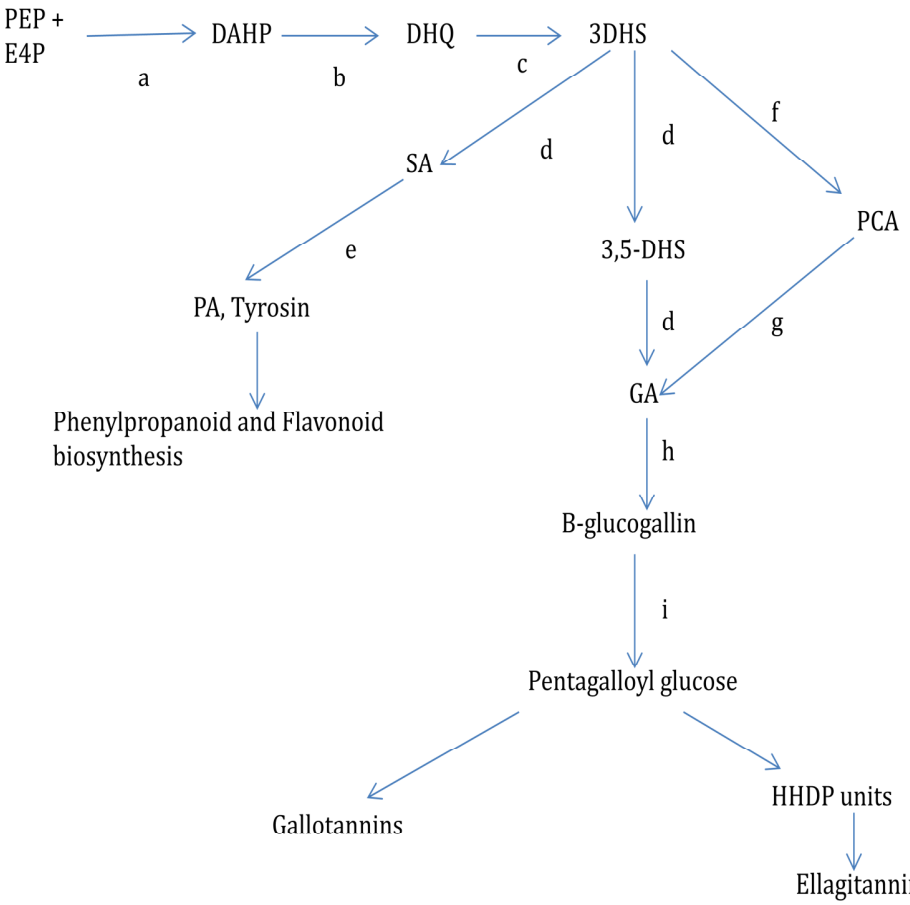



**Figure 2.1** Biosynthesis of gallic acid and phenylpropanoids.Abbreviations of biosynthetic intermediates: phosphoenolpyruvate (PEP), D-erythrose-4-phosphate (E4P), 3-deoxy-D-arabino–heptulosonic acid-7-phosphate (DHAP), 3-dehydroquinic acid (DHQ), 3-dehydroshikimic acid (2-DHS), protocatechuic acid (PCA), shikimic acid (SA), phenylalanine (PA), gallic acid (GA), and hexahydroxydiphenoyl (HHDP) units. Enzymes are: a) DAHP synthase; b) DHQ synthase; c) DHQ dehydrogenase; d) shikimate dehydrogenase; e) shikimate dehydrogenase; f) DHQ dehydrase; g) PCA hydroxylase; h) 1-*O*-galloyl transferase; and, i) galloyl transferase.

The common anthocyanins found in strawberry are cyanidin which has a di-hydroxylated β ring at the 3' and 4' positions produced by F3'H, and pelargonidin, a 4' hydroxylated flavonoid produced by F3H. Flavonol synthase (FLS) catalyzes the dehydrogenation of 3-hydroxyflavonols to corresponding flavonols, quercetin and kaempferol. Dihydroflavonol 4-reductase (DFR) provides the entry step for anthocyanin production using dihydrokaempherol or dihydroquercetin as a substrate resulting in the formation of the leucoanthocyanidins, leucopelargonidin from dihydrokaempherol and leucocyanidin from dihydroquercetin. The leucoanthocyanidins are converted to anthocyanidins by leucoanthocyanidin dioxygenase (LDOX)/anthocyanidin synthase (ANS). Anthocyanins are glycosylated by the UDP:flavonoid-*O*-glucosyltransferase (UFGTs) enzymes, or rhamnosyl transferase (RT). Methylation by *O*-methyl transferase (OMT) may be required to create stable anthocyanidin glucosides that result in pigmentation (Siddiq *et al.*, 1994).

The flavan-3-ols, (+) catechin and (-) epicatechin, are synthesized by two different pathways using a common precursor, leucoanthocyanidin (flavan-3,4-diol). Leucoanthocyanidin can be converted to the 2'3' *trans*-flavan-3-ols, (+) catechin, from direct reduction of leucoanthocyanidin by leucoanthocyanidin reductase (LAR). (-) Epicatechin is produced via two biosynthetic steps using anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR). Proanthocyanidins are oligomers of flavan-3-ols, and result from the sequential addition of starter and extension units of catechin and epicatechin, respectively (Dixon *et al*., 2005).

### 2.4 Regulation of gene expression in flavonoid biosynthesis

#### 2.4.1 Transcriptional regulation

Regulatory genes control the expression of the structural genes. Evidence for this regulation can be obtained either indirectly, via enzyme assays, or directly by mRNA assays of structural genes. In plants, regulation of the flavonoid pathway is largely at the level of transcription of the structural genes, encoding the enzymes for each step of the pathway. transcription factors (TF). Three families of regulators, namely R2R3 MYB, basic helix–loop–helix (bHLH), and WD-repeat (WDR; tryptophan-aspartic acid (W-D) dipeptide repeat) proteins (the MYB-bHLH-WD40 "MBW" complex) are known to coordinately

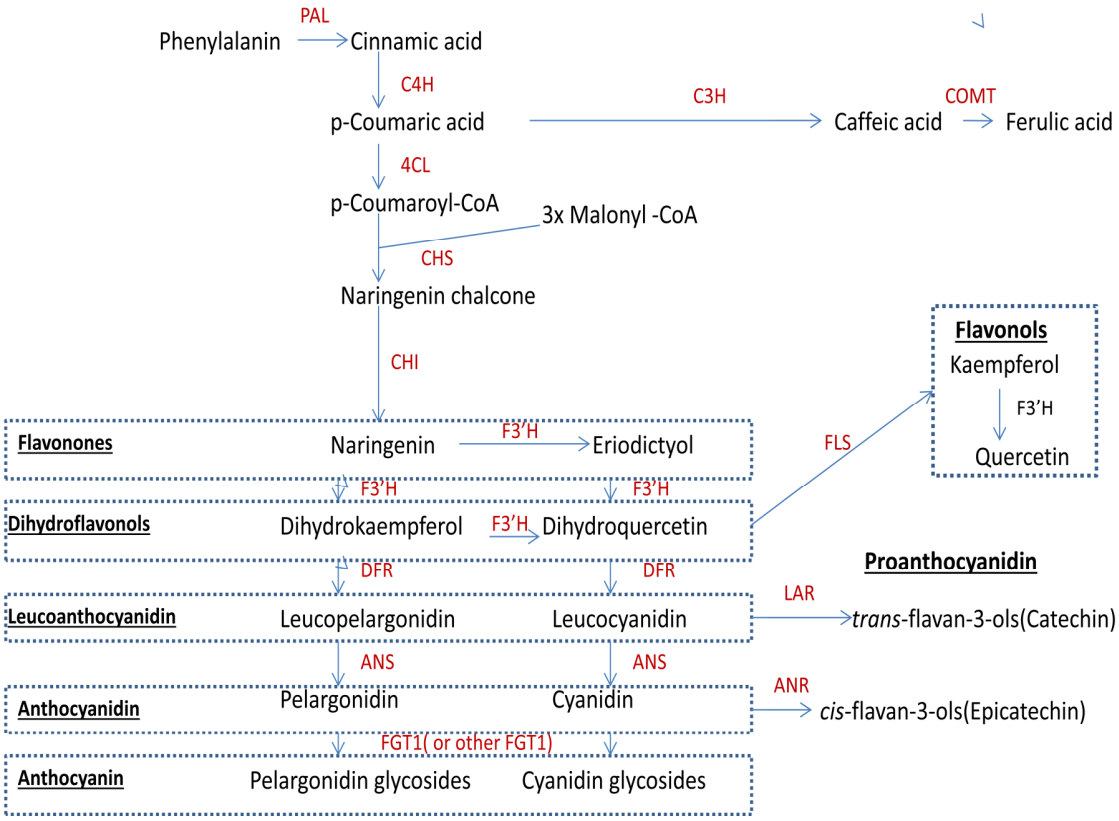



**Figure 2.2** Phenylpropanoid/flavonoid biosynthetic pathway.Abbreviations of biosynthetic enzymes: phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), hydroxycinnamate CoA ligase (C4L), *p*-coumaroyl shikimate/quinate-3'-hydroxylase (C3'H), caffeic acid/5-hydroxyferulic acid–*O*-methyltransferase (COMT), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3'-hydroxylase (F3'H), flavonol synthase (FLS), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin reductase (LAR), anthocyanin reductase (ANR), anthocyanidin synthase (ANS), and UDP:flavonoid- *O*-glucosyltransferase (UFGTs/FGTs).

regulate the structural genes of the flavonoid biosynthetic pathway (Patra *et al*., 2013). MYB-TFs have a structurally-conserved DNA binding domain, also known as a MYB-domain, that can be classified into three subfamilies based on the number of imperfect repeats, including R3 MYB (MYB1R) with one repeat, R2R3 MYB with two repeats, and R1R2R3 MYB (MYB3R) with three repeats (Rosinski *et al.*, 1998; Jin *et al*., 1999). TFs activate or repress the expression of the target genes by recognizing specific *cis*-regulatory sequences in the promoters of the structural genes, and/or by interacting with co-factors to form transcriptional complexes, which bind to promoters of the target genes (Koes *et al.*, 2005; Yang *et al*., 2012).

Two families of regulators, the bHLH (also known as MYC) and MYB proteins, are conserved for the anthocyanin and proanthocyanidin pathway (Koes *et al*., 2005). Production of 3-deoxyflavonoids (in grasses) and flavonols involves R2R3 MYBs without known bHLH cofactors (Mehrtens *et al*., 2005; Falcone *et al*., 2010). The bHLH cofactors may have some overlapping targets (Zhang *et al*., 2003; Zimmermann *et al*., 2004), involving increased DNA binding affinity of the R2R3 MYB factor (Hernandez *et al*., 2004). In contrast, WD40 can be expressed ubiquitously and may not show any direct involvement in transcriptional activation (Lai *et al*., 2013). R2R3 MYB proteins constitute large families in plants, with 126 and 108 genes in Arabidopsis and grape (*Vitis vinifera*), respectively (Stracke *et al*., 2001; Dubos *et al*., 2010).

Research on flavonoid biosynthesis in several species has indicated that there are several MYB genes specifically responsible for the regulation of anthocyanin biosynthesis genes. In Arabidopsis, the early pathway genes (EPGs) were positively regulated by MYB11, MYB12 and MYB111, whereas late pathway genes (LPGs), specifically DFR, ANS and UFGT, were controlled by MYB75 (PRODUCTION OF ANTHOCYANIN PIGMENTS1; PAP1)/MYB90(PAP2)/MYB113/114 (Stracke *et al*., 2007; Gonzalez *et al*., 2008; Patra *et al*., 2013). In *Vitis vinifera*, VvMYB5a (Deluc *et al*., 2006) and VvMYB5b (Deluc *et al*., 2008) were reported to regulate proanthocyanidin biosynthesis, but the late pathway gene VvUFGT was not regulated by VvMYB5b. Two MYBs, VvMYBA1 and VvMYBA2, which are homologs of Arabidopsis AtMYB75, AtMYB90 and AtMYB113/114, were reported to regulate VvUFGT (Azuma *et al*., 2012). These two MYB-genes were not functional in white grape berries (Boss *et al*., 1996), and a mutation in the promoter region of VvMYBA2 produced a color mutant (white berries) with a loss of anthocyanin biosynthesis in the skin (Kobayashi *et al*., 2004;

Walker *et al*., 2007). Homologs of these regulatory genes, MdMYBA/MdMYB1 and MdMYB10, control red pigmentation of apple (*Malus x domestica*). MYB1 and MYBA regulate anthocyanin accumulation in the skin, and MYB10 regulates the LPGs both in skin and flesh (Takos *et al*., 2006; Ban *et al*., 2007; Chagne *et al*., 2007; Lin-Wang *et al*., 2010). The proanthocyanidin-related structural genes LAR and ANR had related expression patterns during fruit development in blueberry (*Vaccinium corymbosum*), with a parallel expression of VcMYBPA1 during early and late stages of flavonol and anthocyanin accumulation, respectively (Zifkin *et al*., 2012). The expression of UFGT in strawberry and pears was regulated by FaMYB10 and PcMYB10, respectively (Wang *et al.*, 2013; Medina-Puche *et al*., 2014).

Orthologs of MYB10 have been isolated from at least 20 Rosaceae species (Lin-Wang *et al*., 2010). In peach (*Prunus persica*), PaMYB10.1, PaMYB10.2 and PaMYB10.3 gene variants were found (Rahim *et al*., 2014). Homologs of PaMYB10.1 and PaMYB10.2 were isolated in sweet cherry (*P. avium*) where subvariant gene PaMYB10.1-1 of variant MYB10.1 showed higher expression in fruit with higher anthocyanin content and was highly correlated with the expression of PaUFGT, whereas subvariant PaMYB10.1-3 showed low levels of expression (Starkevič *et al*., 2015). In nectarine, MYB10 positively regulated UFGT and DFR promoters, and in peach, MYBPA1 may regulate LAR and ANR expression, whereas MYB15 and MYB123 were suggested as candidates for controlling FLS abundance (Ravaglia *et al*., 2013). Variants in *Fragaria* spp. have not been reported.

In all the *Fragaria* spp. studies to date, the regulation of flavonoid biosynthesis, or more specifically anthocyanin biosynthesis, at the level of transcriptional regulation of structural genes have mostly been studied in *F. x ananassa*. Over-expression of FaMYB10 resulted in elevated accumulation of anthocyanin in root, leaf and fruits of *F. x ananassa* (Lin-Wang *et al*., 2010). FaMYB10 was suggested to regulate all pathway genes from CHS to UFGT in both early and late stages of fruit development, but the expression of FaMYB10 was confined to the strawberry fruit receptacle, and the expression of ANS was probably not controlled by FaMYB10 (Medina-Puche *et al*., 2014). Lin-Wang *et al*. (2014) reported that over-expression of FvMYB10 in *F. vesca* resulted in greatly increased anthocyanin concentration, but the concentration of other flavonoids was not affected except for *p*-coumaroyl glucose. Similar results were reported for *F. x ananassa* (Medina-Puche *et al*., 2014), suggesting FvMYB10 may only act on the anthocyanin branch in strawberry.

FaMYB10 and FvMYB10 may have different roles in regulating ANS as FaMYB10-silenced lines showed no change in their ANS level. In contrast, heavily FvMYB10-silenced lines exhibited downregulation of ANS gene expression along with expression of CHS, F3H, DFR and UFGT (Lin-Wang *et al*., 2014; Medina-Puche *et al*., 2014). Two other regulatory genes, FaMYB9 and FaMYB11, interacted with FaTTG1 to regulate proanthocyanidin accumulation during early stages of fruit development (Schaart *et al*., 2013). The expression of FaANS was influenced by FaMYB5 but not by FaMYB10, although this needs further confirmation.

Most of the MYB TFs act as positive regulators, but some negative regulators have also been characterized. MYB repressors have a consensus sequence, LXLXL, present in the EAR motif that interacts with the regulatory partners (bHLH or WDR) or biosynthetic genes (Salvatierra *et al*., 2013). The ectopic expression of FaMYB1 in *Lotus corniculatus* showed a lower level of ANR and LAR1 transcript in leaves. However, anthocyanin did not accumulate in the leaves (Paolocci *et al*., 2011). Over-expression of FaMYB1, a TF isolated from *F. x ananassa,* in tobacco resulted in lower accumulation of certain flavonoids in tobacco flowers (Aharoni *et al*., 2001). However, there was no change in anthocyanin content in FaMYB1-silenced *F. x ananassa* (Medina-Puche *et al*., 2014). Gene expression analysis of FaMYB1 and FvMYB1 during fruit development showed that the highest transcript level for FaMYB1 was at the red, ripe stage, whereas FvMYB1 exhibited little change throughout fruit development (Lin-Wang *et al*., 2010). White-fruited *F. chiloensis* strawberry showed higher expression of MYB1 and downregulation of anthocyanin-related genes (ANS and UFGT) in comparison to its red botanical form (Salvatierra *et al*., 2010; Saud *et al.,* 2009). In contrast to the common red commercial strawberry *F. x ananassa,* FcMYB1 expression level was higher in white *F. chiloensis* at the later stages of fruit development. Suppression of FcMYB1 in white *F. chiloensis* resulted in transcriptional activation of ANS with higher accumulation of pelargonidin-3-glucosides in receptacles, but transcriptional blockage of proanthocyanidin biosynthesis-related genes resulted in no undetectable proanthocyanidin monomers (Salvatierra *et al*., 2013). An interaction between FaMYB1 and bHLH TFs was recently shown to regulate ANS (Schaart *et al*., 2013). Lower expression of ANS was also reported in a white-fruited *Duchesnea indica* in contrast to one with red fruit (Debes *et al.*, 2011).

As noted above, another important constituent of the MBW complex are bHLH factors, which show partially overlapping expression and play redundant roles in the flavonoid biosynthetic pathway, specifically regulating anthocyanin biosynthesis. The

variety of flavonoid-regulating bHLH proteins seems to be much smaller when compared to MYBs. In Arabidopsis, TT8, EGL3 and GL3 are the bHLH proteins reported to date, where EGL3 plays a major role in activation of structural genes for anthocyanin biosynthesis (Stracke *et al*., 2007; Gonzalez *et al*., 2008; Patra *et al*., 2013). Homologs of bHLHs have been reported in apple (bHLH3) and strawberry (bHLH33) (Espley *et al*., 2007; Schaart *et al*., 2013). bHLH33 of apple activated the Arabidopsis DFR promoter conjugated to the MYB10 gene in tobacco, inducing anthocyanin synthesis (Ban *et al*., 2007; Dubos *et al*., 2010). The involvement of bHLH3 activation of UFGT and LAR along with MYB10 and MYBPA1, respectively, was shown in peach (Ravaglia *et al*., 2013). In sweet cherry, bHLH3 showed stable expression throughout fruit development, but the expression of bHLH33 gradually decreased with the later stages of anthocyanin accumulation (Starkevič *et al*., 2015).

### 2.4.2 Hormonal and light regulation

Environmental and hormonal signals play crucial roles in the regulation of fruit development and maturity. In climacteric fruits, such as tomato and apple, ethylene is produced at the onset of ripening along with a respiratory burst. In non-climacteric fruit such as grape and strawberry, an increase in ethylene is rarely observed (Chervin *et al*., 2004). Accumulation of abscisic acid (ABA) has been reported at the onset of ripening in cherry (*Prunus avium*), grape and blueberry (Kondo and Inoue, 1997; Owen *et al*., 2009; Zifkin *et al*., 2012). A coordinated coordination between ABA accumulation and ABA related gene expression during grape berry ripening has been reported (Wheeler *et al*., 2009). In strawberry, high auxin levels promoted fruit growth in the early stages of fruit development but the level declined as the fruit matured (Archbold and Dennis, 1984, 1985; Given *et al*., 1988, Fait *et al.*, 2008). In contrast, ABA levels in strawberry were higher at the later stages of fruit development. The fruit ripening process may be triggered by a defined ABA/auxin ratio in the receptacles of strawberry (Perkins-Veazie, 1995; Jiang and Joyce, 2003). ABA has been shown to participate in regulation of anthocyanin biosynthesis in grape and blueberry (Peppi *et al*., 2008; Zifkin *et al*., 2012). ABA accumulation, and expression of the key gene in the biosynthetic pathway, VcNCED1, was correlated with anthocyanin accumulation in blueberry (Zifkin *et al*., 2012). Anthocyanin production was inhibited when RNAi-mediated silencing of an ABA biosynthetic gene (FaNCED1) and putative ABA receptor gene (FaCHLH) was introduced in strawberry (Jia *et al*., 2011). Medina-Puche *et al*.

(2014) suggested that both ABA content and auxin content of the fruit receptacle of strawberry regulates the expression of FaMYB1 expression during fruit development.

Extensive studies have been carried out on the effect of light on the composition of flavonoids in fruits (Zoratti *et al*., 2014). Flavonoid biosynthesis has been shown to be influenced by light in many species including grape (Spayd *et al*., 2002; Azuma *et al*., 2012), cranberry (*Vaccinium macrocarpon* (Zhou and Singh, 2004), raspberry (*Rubus idaeus*) (Wang *et al*., 2009), apple (Takos *et al*., 2006; Feng *et al*., 2013), and strawberry (Anttonen *et al*., 2006; Kadomura-Ishikawa *et al*., 2013). Most of these studies were done by using fruit bagging and shading experiments. In apple, there was an upregulation of MdCHS, MdCHI, MdF3H, MdLODX, MdDFR1 and MdUFGT with higher accumulation of anthocyanins, flavonols and total phenolics when shaded fruit were exposed to sunlight (Feng *et al*., 2013). In contrast, bilberry (*Vaccinium myrtillis* L.) fruit were either not affected by light or accumulated more anthocyanins in the shade (Jaakola *et al*., 2004). Response to environmental cues may differ depending on the types of flavonoids. Flavonols and proanthocyanidins were more sensitive to environmental factors than anthocyanins in strawberry (Carbone *et al*., 2009). Anthocyanin accumulation by white varieties of grape (Kobayashi *et al*., 2004; Walker *et al*., 2007) and strawberry (Salvatierra *et al*., 2010) were not affected by light.

In recent years, the mechanism of light regulation of the flavonoid biosynthetic pathway was proposed to be due to effects on the MBW complex. In apple, the transcription factors MdMYB1/MdMYBA may act as positive regulators of anthocyanin biosynthesis (Takos *et al*., 2006). MdMYB1 accumulates in light but was degraded in the dark via the 26S proteasome pathway. The bZIP transcription factor may have a direct effect on the regulatory TFs R2R3 MYBs, whereas the ubiquitin E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) may act as a light-induced switch and degrade various photomorphogenesis-promoting transcription factors by the Ub-proteasome system. The ELONGATED HYPOCOTYLE (HY5), a bZIP transcription factor, may be a target of COP1 in darkness and lead to ubiquination and degradation via the 26*S* proteasome pathway (Vierstra *et al*., 2009). However, contradictory results have been reported. The MYB repressor of grape VvMYb4 had no effect on the light response (Matus *et al*., 2009; Azuma *et al*., 2012). In strawberry, a high expression of FvMYB10 was found in petals of flowers but FvMYB1 had no relationship to the light response (Lin-Wang *et al*., 2010).

The quality of light, specifically shorter wavelengths like blue and UV light (< 400 nm), had a positive effect on accumulation of flavonoids. An increased expression of the FaCHS gene was seen in strawberry after 4 d of blue light treatment (Kadomura-Ishikawa *et al*., 2013). Anthocyanin content was increased with an elevated expression of the phototropin FaPHOT2 coordinated with the perception of blue light. The quality of light may also alter the anthocyanin content of the fruit (Kadomura-Ishikawa *et al.*, 2013). Xu *et al*. (2014a) reported an increase in anthocyanin content in strawberry after 4 d of blue light treatment with increased transcript levels of the phenylpropanoid and flavonoid biosynthetic genes PAL, C4H, 4CL, CHS, DFR, ANS and UFGT.

## 2.5 Interaction between phenolic compounds and *Colletotrichum* spp. in strawberry

### 2.5.1 Preformed and induced antifungal compounds

Plants encounter a plethora of potential pathogens in nature, and defense mechanisms conferring tolerance or resistance to these antagonists have evolved that restrict invasion and colonization of the pathogen inside the plant (Ballhorn *et al*., 2009). Plants produce a wide array of secondary metabolites, including phytoalexins, which act as structural barriers, modulators of pathogenicity, and signaling molecules (Hammerschmidt, 2005). The type and origin of these compounds depends on the activation of specific biochemical pathways and may be synthesized around damaged tissue (Boller and Felix, 2009; Veitch, 2009). Preexisting or induced compounds, produced before or after the pathogen attack, respectively, play a key role in this plant defense. Several preformed secondary metabolites (also known as phytoanticipins) may be present at higher concentrations representing an innate chemical barrier to a biotic stress (Lattanzio *et al*., 2006). If the preexisting phenolics are not sufficient for resistance to pathogen infection, plants generally induce the synthesis of phenolics which may be substrates for polyphenol oxidase (PPO) catalyzing the oxidation and production of fungitoxic quinones (Lattanzio *et al*., 2006). Pattern reorganization receptors (PRR) situated on cell surfaces recognize microbe-associated molecular patterns (MAMPs) and activate basal immunity first, then a hypersensitive response such as formation of necrotic spots around infection sites due to programmed cell death to limit the spread of damage within host plants, known as effector-triggered immunity (Lattanzio *et al*., 2006; Boller and Felix, 2009).

The distribution of preformed phenolics can be tissue specific (Lattanzio *et al*., 2006), and studies show varying structure-activity relationships between specific phenolics and resistance to pathogens. Onion varieties resistant to onion smudge disease showed lower spore germination of *Colletotrichum circinas* in the presence of sufficient amounts of preformed catechol and procatechuic acid (Walker and Stahmann, 1955). A small amount of proanthocyanidin and dihydroquercetin were sufficient for resistance to growth of *Fusarium* in a barley mutant (Skadhauge *et al.,* 1997). Inhibition of *Phytophtora* spp. growth was found with phenolic extracts of olive rich in tyrosol, catechin and oleuropein (Del Rio *et al*., 2003). *In vitro* studies with naringenin, kaempferol, quercetin and dihydroquercetin and the rice pathogen *Pyricularia oryzae* showed significant inhibition with naringenin and kaempferol, whereas quercetin and dihydroquercetin had lesser effects (Padmavati *et al*., 1997).

### 2.5.2. Anthracnose fruit rot

Fruits and vegetables are considered rich sources of preformed polyphenols, and yet they are susceptible to attack by some pathogens. The susceptibility of fruit has been associated with physiological changes during fruit ripening, including fruit firmness, pH, cell wall composition, soluble sugars, and secondary metabolites (Sacher, 1973; Brady, 1987; Chillet *et al*., 2007; Moral *et al*., 2008). Anthracnose fruit rot is one of the most economically serious diseases for strawberry production worldwide. The disease is primarily caused by *Colletotrichum acutatum* Simmonds (Bailey *et al*., 1992). Other species such as *C. gleoesporoides* and *C. fragariae* Brooks may be associated with fruit rot. *C. acutatum* can also affect flowers, leaves, petioles, crowns and roots causing blossom blight, defoliation, crown and root rot, and can turn more severe when it causes fruit rot. The disease has been reported to cause 80% plant death in nurseries and yield losses exceeding 50% in well-managed strawberry production fields when the conditions are favorable for disease development (Sreevasanprasad and Talhinhas, 2005). The disease was initially thought to be a “Southern” problem in the United States, favored by warm and moist regions like Florida and Gulf Coast states, but over the past few years it has also caused serious problems in California, New York, Massachusetts, Pennsylvania, Ohio, and as far north as Ontario, Canada.

A number of chemical control measures are considered effective against anthracnose fruit rot in strawberry. Strobilurin fungicides such as azoxystrobin and pyroaclostrobin effectively control fruit disease when sprayed as a standard fungicide (Wharton and Dieguez-Uribeondo, 2004; Mertely *et al*., 2004; Turechek *et al*., 2006). *Colletotrichum* can also be controlled by copper compounds, dithiocarbamates, benzimidazole and triazole compounds (Wharton and Dieguez-Uribeondo, 2004). Repeated and higher rates of fungicides applications have been necessary throughout the season to maintain protection until dry weather suppresses the disease. However, fungicide resistance within *Colletotrichum* has been found (Chung *et al.*, 2010), so multiple strategies to manage it are needed. Planting resistant cultivars is the most logical and effective way to control anthracnose fruit rot disease. A number of resistant cultivars such as Sweet Charlie, Florida Radiance, and Florida Elyana have been bred that reduce the incidence of pre-harvest disease in the field (Mertely *et al*., 2004), but these cultivars are adapted to the Florida climate and do poorly in more northern regions. Biological controls may have potential. Freeman *et al*. (2004) reported that isolates of *Trichoderma* (T-39, T-105, T-161 and T-166) were effective at controlling anthracnose (*Colletotrichum acutatum*) and grey mold (*Botrytis cinera*).

### 2.5.3. Symptoms, pathogen biology and infection process

*Colletotrichum* can affect fruit in two ways, causing pre-harvest disease on immature fruit in the field or post-harvest disease affecting mature fruits at harvest or during storage, but these depend on host specificity and favorable conditions (Wharton and Dieguez-Uribeondo, 2004). Symptoms on fruit appear as whitish, water-soaked lesions up to 3 mm in diameter, which eventually enlarge and become sunken and light tan to dark brown. Anthracnose lesions are smaller on green fruits than on ripening fruit. The lesions may be covered by sticky, pink to orange ooze consisting of masses of spores (conidia) in a mucilaginous matrix (Ellis and Erincik, 2008). Infected fruit may dry down to form hard, black, shriveled mummies. *C. acutatum* also produces irregular leaf spots with dark brown to black irregular lesions on leaf margins. The infected leaves serve as a source of inoculum for flower blight and fruit rot (Peres *et al*., 2005). Dark elongated lesions appear on petioles and runners, which are sometimes girdled by lesions. Affected flowers turn brown and dry quickly, giving plants a blighted appearance. Conidia or appressoria may be washed down

from the upper part of the plants to crowns and roots, causing rot which appears as severe stunting and eventually death of the plant (Peres *et al*., 2005; Ellis and Erincik, 2008).

*C. acutatum* Simmonds was first reported by Simmonds (1965) as a cause of anthracnose of *F. x ananassa*. Wide host ranges of *Colletotrichum* species in different geographical regions and association of several *Colletotrichum* spp., such as *C. gloeosporioides* and *C. fragariae* Brooks, in a single host has led to confusion in identification of *C. acutatum* as a separate species. *C. acutatum* may be considered as a group species named "*Colletotrichum acutatum sensu lato*" and have been identified from diverse hosts and different geographical regions (Lardner *et al*., 1999). Morphological characters, pathogenicity tests, physiological and biochemical approaches and molecular traits (Freeman and Katan, 1997; Simmonds, 1965; Smith and Black, 1990) have been used to classify *Colletotrichum* species. The presence or absence of setae has also been used to aid in the identification of *Colletotrichum* species (Gubler and Gunnell, 1991). *C. acutatum* and *C. gloeosporioides* are morphologically very similar and isolates show variability in culture. Thus, conidia of *C. acutatum* isolates were eliptical to fusiform on strawberry leaf agar (SLA) and mature setae were brown to dark brown, tapered, generally aseptate, did not produce conidia, and were generally shorter than *C. gloeosporioides.* On potato dextrose agar, the colony color of *C. acutatum* was white for 4-5 days but later became gray brown *(*Xie *et al.,* 2010). *C. acutatum* colonies can be pink to orange in color (Wharton and Dieguez-Uribeondo, 2004). The sexual stage of *C. acutatum* has been characterized under laboratory conditions but not found in nature and designated as *Glomerella acutatum* (telomorph) (Guerber *et al*., 2001). In contrast, *C. gloeosporioides* (teleomorph: *Glomerella cingulata*) could be differentiated by gray or olive-gray colonies, dark gray to dark olive in reverse, cylindric conidia, and asci in culture. Isolates of *C. fragariae* showed cylindric conidia, beige to olive to dark gray colonies, and no asci in culture. The presence or absence of setae can be a determining factor in the identification of *Colletotrichum* species (Gubler and Gunnell, 1991).

Disease infection was greater with increased duration of wetness and higher temperature in mature fruit in comparison to immature fruit (Wilson *et al*., 1990). The optimum temperature for infection is between 77 to 86 $^{o}$F (Ellis and Erincik, 2008). The interactions between *Colletotrichum* spp. and hosts are mainly by 1) intercellular hemibiotrophy, and 2) subcuticular intramural necrotrophy depending on the specific host

and tissue type. With strawberry, conidia of *C. acutatum* germinate to form appressoria on all tissue surfaces and penetrate the host tissue by an emerging penetration peg, entering the cuticle, cell wall and cell where an infection vesicle forms. Penetration mycelia grow and establish a subcuticular intramural necrotrophy, avoiding any intimate cytoplasmic interaction with the host cell (Curry *et al*., 2002). With other fruit crops, *Colletotrichum* spp. mostly follow hemibiotrophic interaction, establishing a quiescent infection (biotrophic phase) after formation of appressoria on a fruit surface. Necrotrophic colonization occurs when fruit ripen (Wharton and Schilder, 2003). With strawberry, the biotrophic phase is normally very short.

Strawberry is a soft fruit which shows changes in the synthesis of wide range of phenylpropanoid/flavonoid compounds during fruit development. These compounds may contribute to the plant defense as pre-formed chemical barriers (Amil-Ruiz *et al.*, 2011). Very little work has been done on the possible role of these phenolic compounds on inhibition of *Colletotrichum* isolates from strawberry. Vincent *et al*. (1999) reported a higher amount of pre-formed antifungal compounds in the leaves of moderately anthracnose-resistant commercial strawberry cultivars over susceptible ones, with approximately 15 times more antifungal activity against *Colletotrichum fragariae* though the identity of these compounds were not determined.

### 2.6 Plan of Research

The polyphenolic content of white cultivars of *F. vesca* has not been compared. Due to the unknown origin of all but one white-fruited mutant of *F. vesca*, it was assumed that each resulted from independent mutation events. Because differences in hydroxycinnamic acids were observed in comparison of red versus white *F. chiloensis* (Cheel *et al*., 2005), it was hypothesized that each white *F. vesca* would express a unique polyphenolic profile. Thus, this research was guided by four hypotheses.

**Hypothesis 1.** The white cultivars of *F. vesca* will possess differing total contents of major polyphenolic pools and antioxidant activities.

**Hypothesis 2.** The white cultivars of *F. vesca* will reveal different phenylpropanoid/flavonoid allocation patterns.

**Hypothesis 3.** A white *F. vesca* genotype will exhibit a different metabolite profile and gene expression pattern during fruit development than a red *F. vesca* genotype.

**Hypothesis 4**. Some but not all phenolic compounds found in strawberry will inhibit the growth of *Colletotrichum* spp. *in vitro*.

## Chapter 3: Antioxidant Activity and Phenolic Content of Red- and White-Fruited Genotypes of *Fragaria vesca* and *Fragaria x ananassa*

### 3.1 Introduction

Strawberry is one of the most popular fruit, widely appreciated for its characteristic aroma, unique taste and bright red color. The most important commercial species of strawberry worldwide is *Fragaria x ananassa* Duch., and it is considered a rich source of phytochemicals. The phytochemicals in strawberry are responsible for their health-beneficial antioxidant activity, which can largely be attributed to polyphenols such as anthocyanins and other flavonoid compounds (Cao *et al*., 1996; Wang *et al*., 1996). These compounds can retard or inhibit oxidation of enzymes, proteins, DNA and lipids as metal chelators and scavengers of free radicals (Russo *et al*., 2000). The generation of free radicals and reactive oxygen species (ROS) beyond the endogenous antioxidant capacity of a biological system gives rise to oxidative stress during development of cardiovascular disease, cancer and aging (Dai *et al*., 2010). There is an inverse relationship between strawberry consumption and the risk of cardiovascular diseases and cancer (Hannum, 2004; Pajk *et al*., 2006; Itoh *et al*., 2009; Henning *et al*., 2010; Dai and Mumper, 2010).

For the past several years, increasing attention has been given to the variation in phytochemical content, specifically in phenolic-derived compounds, among genotypes of commercial strawberry (Meyers *et al*., 2003; Scalzo *et al*., 2005; Cheel *et al*., 2007; Panico *et al*., 2009). The varying phenolic and flavonoid compound levels contributed to the variation in antioxidant activity (Meyers *et al*., 2003; Rekika *et al*., 2005; Panico *et al*., 2009). In addition to genotype, cultural practices, environmental factors, maturity, post-harvest handling, and processing impact berry flavonoid content and antioxidant activity (Howell *et al*., 2001; Wang and Zheng, 2001; Wang *et al*., 2002)

Due to the interest in phenolic and flavonoid content of strawberry, effective strategies to increase this content at the molecular level would be valuable. A critical aspect of this effort is development of a clear understanding of regulation of the enzymatic pathway(s) involved in flavonoid production. Coincident with this interest, the diploid wild strawberry species *Fragaria vesca* has emerged as the model system for the octoploid *F. x*

*ananassa* and possibly other members of the Rosaceae family, and its relatively simple genome was recently sequenced (Shulaev *et al*., 2011). However, there have only been a few reports identifying and/or quantifying the phenolic and flavonoid compounds present in *F. vesca* genotypes (Cheel *et al*., 2007; Jabłońska-Ryś *et al*, 2009; Najda *et al*., 2014; Yildiz *et al*, 2014; Dyduch-Sieminska *et al*., 2015). The availability of naturally-derived color mutant genotypes of *F. vesca* with white berries, visual evidence of possibly significant changes in their flavonoid content, provides an angle to explore if the mutation(s) leading to loss of anthocyanins has a meaningful impact on the content of the major pools of polyphenols and antioxidant activity. Given the unknown origin of the majority of white-fruited *F. vesca*, it was hypothesized that each had a unique mutation which would affect the polyphenol content and antioxidant activity in unique (i.e., different) ways. Thus, total phenolics, total anthocyanins, total flavonoids, total proanthocyanidins, and total antioxidant activity of white *F. vesca* genotypes was characterized and compared.

## 3.2 Materials and Methods

### 3.2.1 Chemicals and reagents

Sodium nitrite, sodium carbonate, (+)-catechin, ascorbic acid, citric acid, ferric chloride, Folin-Ciocalteu (FC) reagent, 2, 4, 6-tris (2-pyridyl) -1, 3, 5-triazine (TPTZ), vanillin reagent, hydrochloric acid and acetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Aluminum chloride, sodium hydroxide, gallic acid, methanol and ethanol were purchased from Fisher Scientific (Pittsburgh, PA).

### 3.2.2 Plant material

Five genotypes of white-fruited *F. vesca* were used in the study: Baron Solemacher, Yellow Wonder, Pineapple Crush, Ivory, and White Soul. For comparison, a red-fruited *F. vesca* genotype Baron Solemacher, a red-fruited *F. x ananassa* Earliglow and white-fruited *F. x ananassa* White Pine were also included. Baron Solemacher and White Pine are older German cultivars, Pineapple Crush is also likely from Europe originally, and White Soul is likely White (or Weiss) Solemacher, also an old German cultivar. Earliglow is a common commercial variety. The origin of Yellow Wonder and Ivory are not known. White Baron Solemacher is a product of a gamma irradiation-induced mutation of red Baron Solemacher.

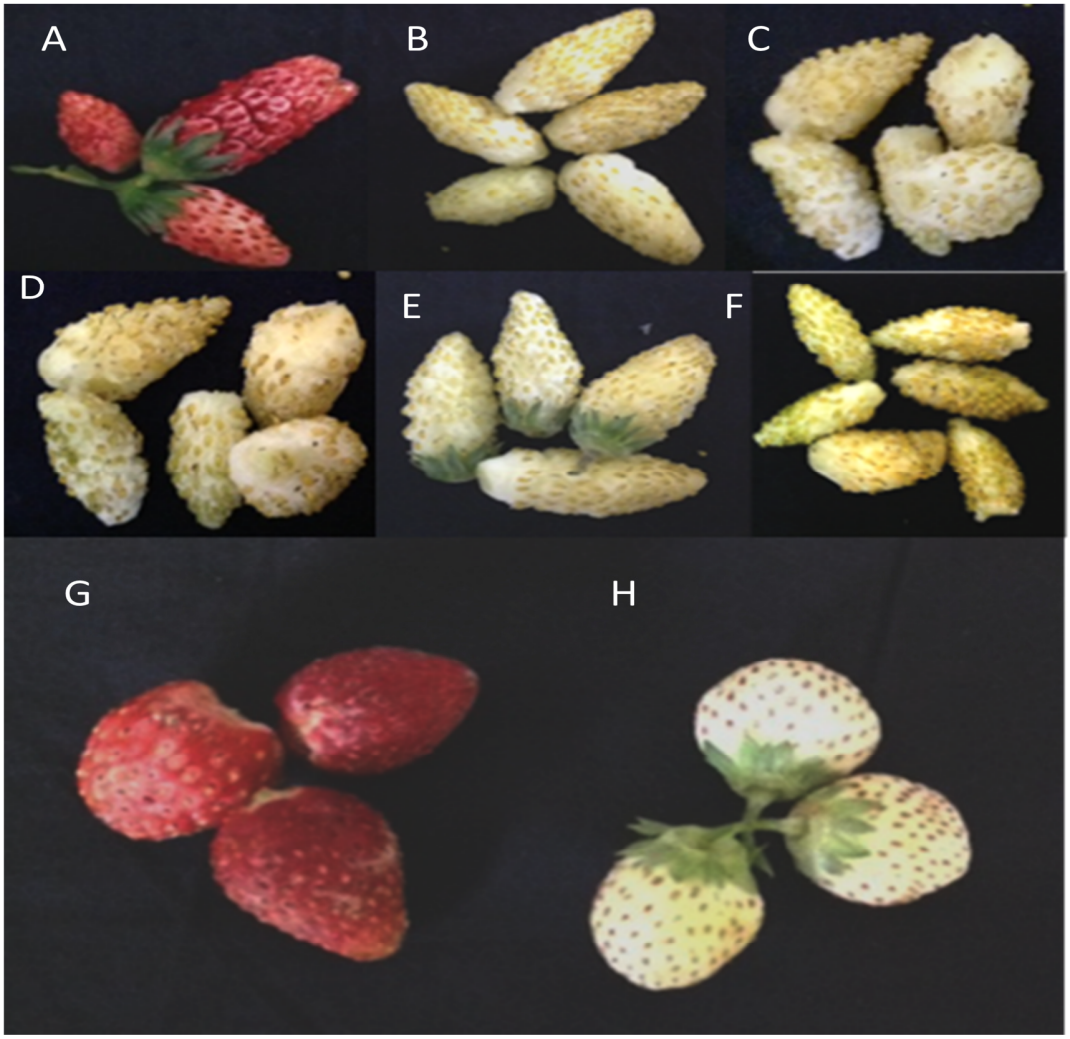



**Figure 3.1** Ripe berries of red and white *F. vesca* and *F.* x *ananassa*.From top left to right: *F. vesca,* A) Baron Solemacher (red) (BSr), B) Baron Solemacher (white) (BSw), C) Yellow Wonder (white) (YW); middle left to right: D) Pineapple Crush (white) (PCw), E) Ivory (white), (Iw), F) White Soul (white) (WSw); *F. x ananassa*, bottom left to right G) Earliglow (red) (Er), and H) White Pine (white) (WPw).

Eight or more plants of each cultivar were grown in 1.5-L containers in MetroMix 360 (Scotts, Marysville, OH, USA), and were watered and fertilized as needed. The plants were grown outdoors from March to November and in greenhouses from November to March in Lexington, KY. Fruit were harvested throughout the year when fully-colored (red fruit only) and/or when softening commenced (red and white fruit). Due to limited plant number and low yield per plant, fruit were harvested as they were available, and were combined within harvest intervals of variable length and across plants within a genotype until sufficient biomass had been collected. Upon harvest, fruit were immediately frozen in liquid $N_2$ and stored at -80$^{o}$C until further use.

### 3.2.3 Fruit extraction

To extract fruit, a 12 g of sample of each genotype was homogenized in a Waring blender after mixing with 15 mL of 80% methanol. There were three replicate extractions of each genotype, with each replicate from a separate harvest interval. After filtration by Buchner funnel with Whatman No. 42 filter paper, the solution was then filtered through a Gelman Laboratory 0.45 µm Acrodisc LC PVDF syringe filter.

### 3.2.4 Quantification of total anthocyanins (TA)

Total anthocyanins were quantified following a modified version of the Glories' method (Fukomoto amd Mazza, 2000). Briefly, 0.25 mL of fruit extract for each type of fruit was mixed with 0.25 mL of 0.1% HCL in 95% ethanol and 4.55 mL of 2% HCL. The absorbance of the solution was then read at 520 nm in the spectrophotometer (Cary 50, Varian, Walnut Creek, Calif.). TA was expressed as mg of cyanidin-3-glucoside /100 g fresh weight (FW).

### 3.2.5 Quantification of total phenolics (TP)

Total phenolics were determined by the Folin-Ciocalteu method (Singleton *et al*., 1965) with some modification. A 0.2 mL aliquot of fruit extract was mixed with 1 mL of Folin-Ciocalteu phenol reagent and 0.8 mL of 7.5% of sodium carbonate and incubated for 40 min at room temperature. The absorbance of the mixture was then measured at 765 nm. TP was expressed as mg of gallic acid equivalents (GAE)/100 g FW.

### 3.2.6 Quantification of total flavonoids (TF)

Total flavonoid content was evaluated by the aluminum chloride colorimetric assay (Zhishen *et al*., 1999). A 1 mL of aliquot of strawberry extract was added to 4 mL with Millipore-purified water followed by the addition of 0.3 mL of 5% $NaNO_2$ solution. After 5 min at room temperature, 0.3 mL of 10% $AlCl_3$ was added. At 6 min, 2 mL of 1M NaOH was added, and total volume was made up to 10 mL with Millipore-purified water. The solution was mixed thoroughly, and absorbance was measured at 510 nm. Total flavonoid content of strawberries was expressed as mg catechin equivalents (CE)/100 g FW.

### 3.2.7 Quantification of total proanthocyanidins (TPA)

Total proanthocyanidin (TPA) content was determined by vanillin assay (Sun *et al*., 1998). A 0.1 to 1 mL aliquot of extract was brought to 1 mL by addition of glacial acetic acid. Then, 5 mL of vanillin reagent consisting of 1% vanillin and 8% HCl in glacial acetic acid (1:1, v/v) was added, and samples were held in a water bath at 30 °C for 20 min. Absorbance was read at 510 nm. Proanthocyanidin content was expressed as mg catechin (CE)/100 g FW.

### 3.2.8 Determination of total antioxidant activity (TAC)

Total antioxidant activity was determined by a modification of the ferric reducing/antioxidant power (FRAP) assays (Arnous *et al*., 2002). The assay was conducted by adding 0.05 mL of diluted fruit extracts to 0.05 mL of 3 mM ferric chloride in 5mM citric acid in a 1.5 mL of Eppendorf tube, and was incubated in a water bath for 30 min at 37 °C. Then, 0.9 mL of 2, 4, 6-tris (2-pyridyl)-1, 3, 5-triazine (TPTZ) solution in 0.05 M HCL was added to the mixture, and the mixture was vortexed and held for 10 min at room temperature. Next, the absorbance of the mixture was read at 620 nm. Results were expressed as mg of ascorbic acid equivalents (AAE)/100 g FW.

### 3.2.9 Statistical analysis

Because the objective was to determine if each *F. vesca* had a unique set of polyphenolic contents and antioxidant activity, each cultivar was considered a unique genotype for statistical analyses. Thus, to evaluate significant differences among genotypes,

analyses of variance (ANOVA) were performed (Sigmaplot for Windows, v. 12.0), and genotype means were compared using Fisher's LSD at P=0.05.

## 3.3 Results and Discussion

### 3.3.1 Total anthocyanins

Total anthocyanin content (TAC) content was only measurable in red cultivars of *F. vesca* and *F. x ananassa;* anthocyanins were not detected in the white cultivars of either species (Table 3.1). The anthocyanin content was slightly higher in commercial *F. x ananassa* Earliglow than in the red *F. vesca* Baron Solemacher. A wide variation of total anthocyanin content among red *F. vesca* cultivars was reported by Yildiz *et al*. (2014). For red *F. x ananassa* cultivars, values for total anthocyanins reported by Meyer *et al*. (2003) and Wang and Lewers (2007) were comparable with values in the present study.

### 3.3.2 Total phenolics

Total phenolic (TP) content (Table 3.1) ranged from 107 to 280 mg GAE/100 g FW. Among the *F. vesca* cultivars, the white-fruited Yellow Wonder, Baron Solemacher, and Ivory revealed a higher TP content than the red Baron Solemacher and the two other white genotypes. In contrast, the white cultivar of *F. x ananassa* had a considerably lower TP content than the red Earliglow. Values for the red cultivars of both *F. vesca* and *F. x ananassa* in the present work were comparable to reported values of 123 to 273 mg GAE/100 g FW for *F. x ananassa* cultivars (Meyer *et al*., 2003; Scalzo *et al*., 2005; Wang and Lewers., 2007), and of 165 to 235 mg GAE/100 g FW for *F. vesca* cultivars (Cheel *et al*., 2007; Jabłońska-Ryś *et al*., 2009; Najda *et al*., 2014; Yildiz *et al*., 2014). Comparison of a white to a red cultivar of the octoploid *F. chiloensis* indicated a greater TP content in the white cultivar (Cheel *et al*., 2007), a difference not observed for *F. x ananassa* and with some but not all *F. vesca* cultivars in the present work.

### 3.3.3 Total flavonoids

Ivory, a white *F. vesca*, showed the highest total flavonoid (TF) content (Table 3.1), followed by Baron Solemacher and Yellow Wonder, and all had greater TF content than the red Baron Solemacher. Najda *et al*. (2014) reported that the TF content of two red cultivars

**Table 3.1** Content of total anthocyanin, phenolics, flavonoids and proanthocyanidins of fruit from *Fragaria vesca* and *Fragaria* x *ananassa* genotypes with red and white fruit.

| Cultivar | Berry Color | Total Anthocyanins[a] | Total Phenolics[b] | Total Flavonoids[c] | Total Proanthocyanidins[d] |
|---|---|---|---|---|---|
| | | | *F. vesca* | | |
| Baron Solemacher | red | 54 ± 1 b[f] | 249 ± 6 c | 90 ± 6 c | 133 ± 6 b |
| Baron Solemacher | white | ND | 270 ± 8 b | 119 ± 3 b | 77 ± 1 d |
| Yellow Wonder | white | ND | 280 ± 4 a | 117 ± 4 b | 81 ± 3 cd |
| Pineapple Crush | white | ND | 246 ± 5 c | 91 ± 1 c | 54 ± 1 f |
| Ivory | white | ND | 268 ± 6 b | 129 ± 2 a | 84 ± 6 c |
| White Soul | white | ND | 241 ± 6 c | 86 ± 2 c | 66 ± 4 e |
| | | | *F. x ananassa* | | |

| | | | | | |
|---|---|---|---|---|---|
| Earliglow | **red** | **57 ± 1 a** | **207 ± 5 d** | **68 ± 4 d** | **203 ± 2 a** |

Table 3.1 (continued)

| | | | | | |
|---|---|---|---|---|---|
| White Pine | white | ND | 107 ± 1 d | 49 ± 1 d | 27 ± 1 g |

[a]Total anthocyanin (mg cyanidin-3-glucoside equivalents/100 g FW).

[b]Total phenolics (mg gallic acid equivalents/100 g FW).

[c] Total flavonoids (mg catechin equivalents/100 g FW).

[d] Total proanthocyanidins (mg catechin equivalents/100 g FW).

[e] Total antioxidant activity (mg ascorbic acid equivalents/100 g FW).

[f]Values are means of 3 replicates ± SD. Different letters in each column indicate significant differences by by Fisher's LSD at P=0.05. ND = not detected.

of *F. vesca* was 47 and 56 mg CE/100 g FW, lower than values in the present study for both white and red cultivars. Both the red and white cultivars of *F. x ananassa* had the lowest TF values. Comparable to the present results, Meyer *et al*. (2003) obtained between 46 to 70 mg CE/100 g FW TF for *F. x ananassa* cultivars, close to the present values. In contrast, Cheel *et al*. (2007) found that the *F. x ananassa* cultuivar Chandler had a TF content of 123 mg CE/100 g FW, higher than in our study. The significance of the impact of the environment and cultivation techniques on apparent genotypic differences among the various studies is unknown.

### 3.3.4 Total proanthocyanidins

The red cultivars of both *Fragaria* species had a greater TPA content than the white cultivars (Table 3.1). In addition, the white cultivars of *F. vesca* varied but were all greater than the white *F. x ananassa.* Dyduch-Sieminska *et al*. (2015) reported that total tannin content of Baron Solemacher was greater than that of Yellow Wonder, the opposite of the present results. However, the analytical techniques were not the same.

### 3.3.5 Total antioxidant activity

*F. vesca* fruit had significantly higher TAC than *F. x ananassa* fruit (Figure 3.1). Halvorsen *et al.* (2002), Cheel *et al*. (2007), and Yildiz *et al*. (2014) also reported higher TAC values in *F. vesca* fruit than in *F. x ananassa* fruit. Although the white *F. vesca* Yellow Wonder had the greatest antioxidant activity, the white *F. vesca* were not consistently greater than the red *F. vesca*. Yildiz *et al*. (2014) reported wide variation among red *F. vesca* genotypes, and that the TAC values can differ among strawberry cultivars when determined by different antioxidant assays. A positive correlation was observed between total phenolic content and total antioxidant capacity of the genotypes (Figure 3.2). However, there was a positive but weaker association between TF content and TAC ($R^2$ =0.643, $p < 0.05$). These observations were in agreement with the studies of Meyers *et al*. (2003) for several cultivars of *F. x ananassa.*

Strawberries contain numerous phenolic compounds. However, the presence and content of different polyphenols among cultivars may create different antioxidant activities (Macheix *et al.*, 1990). Thus, lacking anthocyanins did not compromise antioxidant activity.

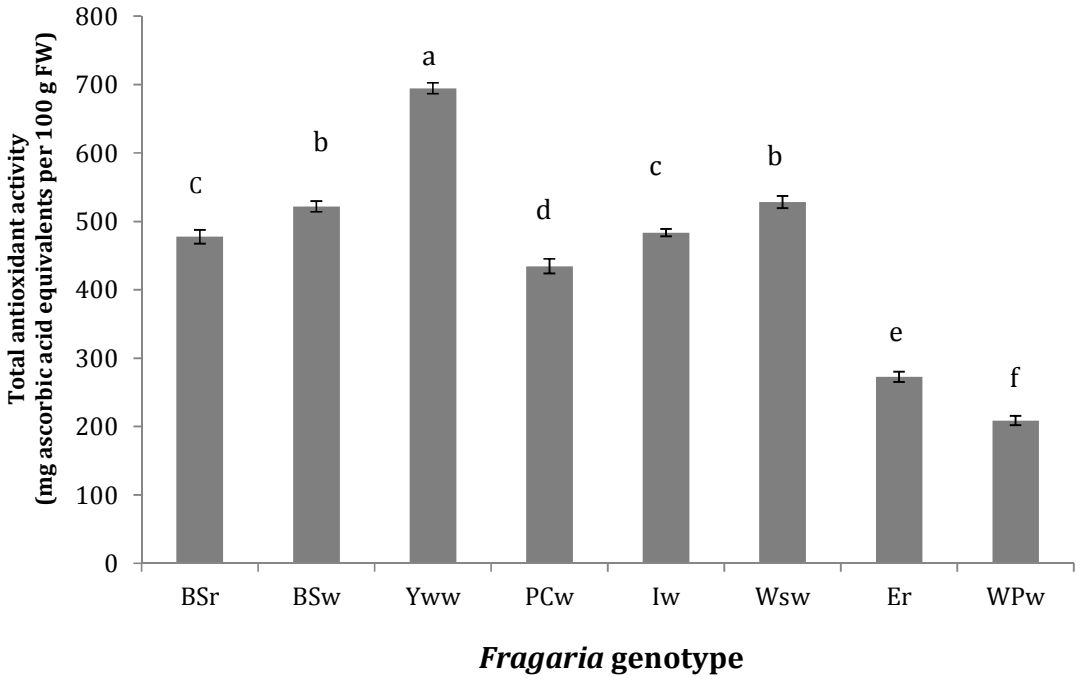



**Figure 3.2** Total antioxidant activity of *Fragaria* genotypes by the FRAP assay.

Values are means ± SD (n=3). Genotypes with red fruit were Earliglow (Er) (*F. x ananassa*) and Baron Solemacher (BSr) (*F. vesca*). Genotypes with white fruit were White Pine (WPw) (*F. x ananassa*) and Baron Solemacher (BSw), Yellow Wonder (YWw), Pineapple Crush (PCw), Ivory (Iw), and White Soul (WSw) (all *F. vesca*). Genotypes with different letters were significantly different by Fisher's LSD at P= 0.05.

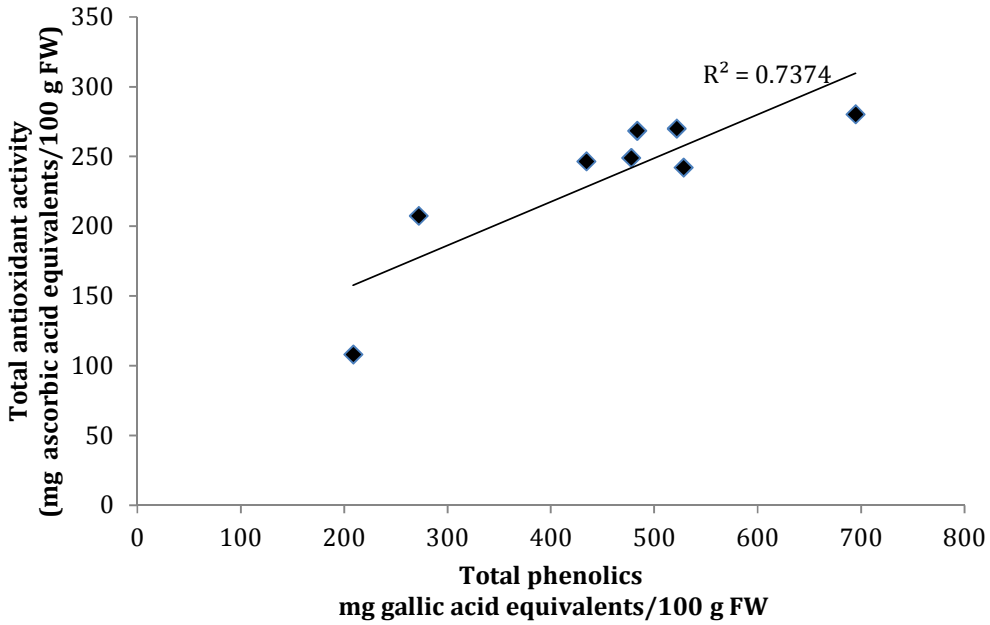



**Figure 3.3** Relationship between total phenolic content and total antioxidant activity.

In *F. vesca,* white strawberries can be considered rich sources of antioxidant activity, at least equal to red strawberries. However, it was also clear that the white *F. vesca* differed from one another, with Pineapple Crush and White Soul more often lower than white Baron Solemacher, Yellow Wonder, and Ivory in each polyphenolic group. The situation for *F. x ananassa* may not be the same, as the red cultivar was consistently higher in all assays than the white cultivar, although a single genotype of each type is too few to be broadly applicable to all *F. x ananassa*. This study provided a preliminary platform for the understanding of overall polyphenolic composition and antioxidant activities of different cultivars of both *Fragaria* spp.

## Chapter 4: Comparative Analyses of Polyphenolic Composition of *Fragaria* spp. Color Mutants

### 4.1. Introduction

Strawberries are a rich source of phenolic acids and polyphenolic compounds including flavonoids, such as the anthocyanins which provide the red color of the fruit. The composition of the commercial strawberry (*Fragaria x ananassa* Duch.) has been extensively studied (Määttä-Riihinen *et al*., 2004; Kosar *et al*.., 2004; Seeram *et al*., 2006; Aaby *et al*., 2007, 2012; Buendia *et al*., 2010; Kajdzanoska *et al*., 2010; Kelebek *et al*., 2011;), and berry content of the major phenolic compounds such as anthocyanins, proanthocyanidins or flavan-3-ols, free and conjugated ellagic acid (EA), and ellagitannins are well-documented. In addition, the polyphenol composition of strawberry fruit has been shown to depend on factors such as genotype (Carbone *et al*., 2009; Doumett *et al*., 2011; Munoz *et al*., 2011), maturity stage (Kosar *et al*., 2004), production site (Josuttis *et al*., 2013), environmental effects (Carbone *et al*., 2009; Bacchella *et al*., 2009; Josuttis *et al*., 2013), and even extraction solvent (Kajdzanoska *et al*., 2011) and analytical method (Buendia *et al*., 2010; Aaby *et al*., 2012).

Flavonoids may play important roles in plant responses to biotic and abiotic stresses (Gould *et al*., 2006), including providing some pathogen resistance (Chappell *et al*., 1984; Dixon and Paiva, 1995). For the consumer, these compounds play multiple roles in nutritional, organoleptic and commercial properties of fruits and vegetables as well (Quideau *et al*., 2011). Flavonoids have shown a wide range of biological effects when consumed, including antioxidant, antimicrobial, anti-inflammatory and vasodilatory activities which reduce the risk of cancer and cardiovascular disease and improve visual activity (Hollman *et al*., 1996; Kaur *et al*., 2001; Sun *et al*., 2002), spurring interest in evaluating their biosynthesis in fruits and vegetables and their functional roles. Polyphenols are synthesized through the shikimate, phenylpropanoid and flavonoid pathways.

The economically important commercial strawberry is octoploid and a hybrid of the wild octoploids *F. chiloensis* and *F. virginiana* (Hancock *et al*., 2008). The wild species *F. vesca,* or Alpine strawberry, is a self-pollinating diploid with a relatively small genome size

(164 Mbp/C), comparable to that of *Arabidopsis thaliana* (Akiyama *et al.,* 2001). Because of its morphological diversity and interfertility (Hancock *et al*., 2008), it has come to serve as a model system for strawberry and other members of the Rosaceae family including apple. The full *F. vesca* genome was recently characterized *(*Shulaev *et al*., 2011). The species includes both common red-fruited types as well as some white-fruited mutants. Because it provides a model system with applicability to a number of valuable commodities within the Rosaceae family, there is a need for a comprehensive understanding of the biosynthesis of the polyphenolic components of *F. vesca*. A study of the polyphenolic composition of *F. vesca* could contribute to an understanding of metabolic flux through the complex, branched flavonoid biosynthesis pathway (Carbone *et al*., 2009), and the white-fruited mutants provide a unique contrast to the red form in such analyses.

Both natural and genetically-engineered mutants provide the opportunity for determining how altering metabolic flux affects the production of flavonoids. Investigating the polyphenolic composition of these mutants could also contribute insight into the regulation of the overall biosynthetic pathway/network of polyphenol biosynthesis in strawberry. Identification and quantification of metabolites of different mutants, independent of genomic data, could allow comparative analyses for determining where alterations in the polyphenol biosynthetic pathway occur and establish a platform for further molecular analyses (Rochfort *et al*., 2005; Sun *et al*., 2014). For example, combined overexpression of the *Arabidopsis* transcriptional regulators AtTT2, AtTT8 and AtttG1 in *F. x ananassa* showed increased proanthocyanidin and decreased anthocyanin in the ripe berry (Schaart *et al*., 2013). Down-regulation of UDP-glucose:anthocyanidin glucosyltransferase (FaGT1) in *F. x ananassa* resulted in re-channeling metabolite flux to flavan-3-ols with a decrease in anthocyanin content (Griesser *et al*., 2008). Polyphenol profiles of a white *F. chiloensis* indicated an increased content of cinnamic acid derivatives (Cheel *et al*., 2005; Simirgiotis *et al*., 2010) due to low expression of cinnamate 4-hydroxylase, leading to a low flux of compounds into the later stages of flavonoid and anthocyanin biosynthesis (Saud *et al*., 2009).

Given the unknown origin of the majority of white-fruited *F. vesca*, it was hypothesized that each had a unique mutation which would affect the polyphenol content and antioxidant activity in unique (i.e., different) ways. The study was performed to compare five white *F. vesca* genotypes, and included a red *F. vesca* and a red- and a white-

fruited genotype of *F. x ananassa* as references for the other major species and color groups, by 1) identifying the major phenolic compounds, and 2) quantifying the content of specific individual target phenolic compounds representing general polyphenolic groups as potential indicators of genotypic differences in phenylpropanoid/flavonoid biosynthesis. Further, the results were analyzed as a first step to clarifying whether all of the white-fruited genotypes share the same mutation(s) resulting in their low anthocyanin content.

## 4.2 Materials and Methods

### 4.2.1 Chemicals

Gallic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, caffeic acid, ellagic acid, (+)-catechin, (+)-epicatechin, quercetin, kaempferol, quercetin-3-β-D-glucoside, quercetin-3-glucoronide, and kaempferol-3-glucoside were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Pelargonidin chloride, pelargonidin-3-glucoside, and cyanidin-3-glucoside were obtained from Extrasynthase S.A. (Genay, France). Ellagitannin in the form of castalagin was kindly provided by Dr. S. Quideau (Université Bordeaux, France). Acetone, acetonitrile, methanol and chloroform were purchased from Fisher Scientific Co. (Tustin, CA). Water was of Milli-Q quality.

### 4.2.2 Plant material

Five genotypes of white-fruited *F. vesca* were used in the study: Baron Solemacher, Yellow Wonder, Pineapple Crush, Ivory, and White Soul. For comparison, a red-fruited *F. vesca* genotype Baron Solemacher, a red-fruited *F. x ananassa* Earliglow and a white-fruited *F. x ananassa* White Pine were also included. Baron Solemacher and White Pine are older German cultivars, Pineapple Crush is likely from Europe originally, and White Soul is likely White (or Weiss) Solemacher, also an old German cultivar. Earliglow is a common commercial variety. The origin of Yellow Wonder and Ivory are not known. White Baron Solemacher is a product of a gamma irradiation-induced mutation of red Baron Solemacher. Eight or more plants of each cultivar were grown in 1.5-L containers in MetroMix 360 (Scotts, Marysville, OH, USA), and were watered and fertilized as needed. All plants were grown outdoors from March to November and in greenhouses from November to March in Lexington, KY. Fruit were harvested throughout the year when fully-colored (red fruit only) and/or when softening commenced (red and white fruit). Due to limited plant number and

low yield per plant, fruit were harvested as they were available, and were combined within harvest intervals of variable length and across plants within a genotype until sufficient biomass had been collected. Upon harvest, fruit were immediately frozen in liquid $N_2$ and stored at -80°C until further use.

### 4.2.3 Extraction of phenolic compounds

Phenolic compounds were extracted from frozen fruit samples (three replicates of 5 g from each cultivar, (~5 fruit per *F. vesca* genotype, and 2 fruit per *F. x ananassa* genotype)) which were first ground to a powder in a mortar and pestle using liquid nitrogen. The powders were extracted with 10 mL of extraction solution (acetone/water/acetic acid; 70:29.5:0.5, v/v/v) three times at 4°C in the dark for 1 h per extraction (Simirgiotis *et al*., 2010), and the extraction volumes were combined. Phenolics were separated from insoluble plant material by vacuum filtering through Whatman No. 1 filter paper. The filtrates were transferred to a separatory funnel, gently mixed with a 2X volume of chloroform and stored overnight at 4°C until a clear partition between the two phases was obtained. The upper aqueous phase was collected and the acetone/chloroform phase was evaporated in an evaporator at 40 °C under vacuum requiring 5 to 10 min (Wrolstad *et al*., 2004). The aqueous residues were filtered through an activated Sep Pack C-18 solid phase column, washed with acidified water (pH 3.4), and the phenolic content was eluted with acidified methanol (pH 3.4). The methanol solutions were evaporated at 37°C under reduced pressure (Buendia *et al*., 2010), and residues were re-dissolved in 2 mL of 100% methanol and filtered through a 0.45 µm nylon filter. Samples were stored at -80 °C until analysis.

### 4.2.4 LC/ESI-MS/MS analysis

The HPLC system consisted of dual Varian ProStar 210 pumps with a Varian ProStar 410 auto injector. Chromatographic separations were carried out on a 100 mm × 2.1 mm, 2.6 µm Phenomenex Kinetex C18 column. The mobile phase consisted of two solvents, water/formic acid (99.9%/0.1%, v/v) (solvent A) and methanol/formic acid (99.9%/0.1%, v/v) (solvent B). A linear gradient was used starting with 5% B at 0 min, to 50% B at 17 min, to 95% B at 22 min, then a return to 5% B at 24 min and held at 5% B for 10 min. The flow rate was 200 µL/min.

The mass detector was a Varian 1200L triple quadruple mass spectrometer with an electrospray ionization (ESI) interface controlled by Varian MS Data review (ver. 6.42) software. A 10 µL sample was injected into the HPLC-MS mobile phase and then directly into the ESI interface without splitting. All replicate samples were run in triplicate, in positive mode, followed by another positive mode, and then a negative mode interrupted by blank (solvent) injection to ensure no carryover between two runs. Nitrogen was used as the drying gas, and the capillary was maintained at 300 °C and voltage of 32V.

The multiple reactions monitoring (MRM) mode was used for the identification of the compounds for which authentic standards were available. The MS/MS parameters were optimized for each compound using direct infusion of flavonoid standards to the ESI-MS/MS system. Analyses were carried out in positive mode for the anthocyanins and in negative mode for the flavan-3-ols, flavonols, ellagitannins and ellagic acid derivatives. Specific parent ions $[M-H]^-$ or $[M+H]^+$ and their corresponding fragment ions were determined for each compound, as well as the appropriate collision energies using argon as the collision gas utilizing collision-induced disassociation (CID). Compounds were identified by comparison of the retention time and mass spectra with pure commercial standards, if available. Mass spectra and fragmentation patterns from our own library and from published literature (Table 4.1) were used for the identification of compounds for which standards were not available. Primarily the MS fragmentation patterns were followed from the literature cited. For these compounds, analyses were also done in the full scan mode with the mass ranging from 150 to 1200 *m/z* to ascertain specific ions of interest. A general range of polyphenolic compounds were obtained by monitoring specific *m/z* values of aglycone standards in the MRM analyses. Once the specific ions of interest were identified, HPLC-MS and MS/MS experiments of the parent ions and most abundant fragment ions were analyzed by a chromatographic run using similar parameters of a closely related authentic standard (Simirigoitis *et al*., 2010).

The basic structure of a flavonoid is the presence of a characteristic aglycone form which undergoes additional hydroxylation, methylation, and, most abundantly, glycosylation, giving rise to different flavonoid derivatives. Retention times are inversely correlated with increasing glycosylation, and the position of glycosylation or methylation also has an effect on retention time (Cuyckens *et al*., 2004; Rak *et al*., 2010). For the present study, derivatives

of ellagic acid and quercetin were fragmented at *m/z* 301 after the loss of a sugar moiety. Comparison with the fragmentation pattern of authentic standards for

**Table 4.1** List of compounds with references used for the identification

| MW | Compounds | *Fragaria* spp.[a] | References[b] |
|---|---|---|---|
| | Hydroxycinnamic acids | | |
| 326 | *p*-Coumaroyl glucose | *Fa, Fc, Fv* | 1,2,3,4,5,7,8,9 |
| 180 | Caffeic acid | *Fv* | 8 |
| 354 | Chlorogenic acid | *Fv* | 8 |
| 342 | Caffeoyl hexose | *Fa* | 4 |
| | Flavonols | | |
| 478 | Quercetin-3-glucuronide | *Fa, Fc, Fv* | 1,2,3,4,5,7,8 |
| 464 | Quercetin-3-glucoside | *Fa, Fv* | 4.8,9 |
| 534 | Quercetin-3-malonylglucoside | *Fa* | 2,7 |
| 506 | Quercetin-acetyl hexoside | *Fa, Fv* | 9 |
| 448 | Kaempferol-3-glucoside | *Fa, Fc, Fv* | 1,4,8,9 |
| 594 | Kaempferol-3-coumaroylglucoside | *Fa, Fc, Fv* | 1,2,3,4,5,7,8 |
| 490 | Kaempferol-3-acetylglucoside | *Fa, Fv* | 4,8 |

Table 4.1(continued)

| | | | |
|---|---|---|---|
| 534 | Kaempferol-malonylglucoside | *Fa* | 2,7,4 |
| | Proanthocyanidins | | |
| 290 | Catechin | *Fa, Fc, Fv* | 1,2,3,4,5,,6,7,8,9 |
| 290 | Epicatechin | *Fv* | 8 |
| 578 | Procyanidin dimer | *Fa, Fv* | 3,4,7,8, |
| 866 | Procyanidin trimer | *Fa, Fv* | 2,3,4,7,8,9,11 |
| 1154 | Procyanidin tetramer | *Fa, Fc, Fv* | 4,5,9,11 |
| 1442 | Procyanidin pentamer | *Fa* | 3,7,11 |
| | Anthocyanins | | |
| 448 | Cyanidin-3-glucoside | *Fa, Fc, Fv* | 1,2,3,4,5,6,7,8,9 |
| 534 | Cyanidin-3-malonylglucoside | *Fa, Fc, Fv* | 2,4,5,7,8,9 |
| 594 | Cyanidin-3-rutinoside | *Fa, Fc* | 1,3,4,5,7, |
| 448 | Cyanidin-3-galactoside | *Fa, Fv* | 6,8 |
| 432 | Pelargonidin-3-glucoside | *Fa, Fc, Fv* | 1,2,3,4,5,6,7,8,9 |
| 519 | Pelargonidin-3-malonylglucoside | *Fa, Fv* | 4,8 |

Table 4.1 (continued)

| | | | |
|---|---|---|---|
| 578 | Pelargonidin-3-rutinoside | *Fa, Fc* | 1,3,4,5,7 |
| 462 | Peonidin-3-glucoside | *Fa, Fv* | 6,8,9 |
| 547 | Peonidin-3-malonylglucoside | *Fv* | 8.9 |
| | Ellagic acid derivatives | | |
| 302 | Ellagic acid | *Fa, Fc, Fv* | 1,2,3,4,5,7,8,9,10 |
| 448 | EA deoxyhexoside | *Fa, Fv* | 2,3,4,7,10 |
| 434 | EA pentoside | *Fa, Fc, Fv* | 2,5,8 |
| 464 | EA hexoside | *Fa* | 4 |
| 448 | Methyl EA pentoside | *Fa, Fv* | 1,8,9 |
| 478 | Methyl EA hexose | *Fv* | 9 |
| 462 | Dimethyl EA pentoside | *Fv* | 9 |
| | Ellagitannins | | |
| 935 | Galloyl-bis-HHDP glucose | *Fa, Fv* | 2,3,4,7,8,9 |
| 1236 | Di-HHDP glucose galloyl EA | *Fa, Fv* | 7,10 |
| 934 | Dimer of galloyl di HHDP glucose | *Fa,Fc* | 2,4,5,7 |

Table 4.1 (continued)

| | | | |
|---|---|---|---|
| 784 | bis HHDP glucose | *Fa, Fc, Fv* | 2,4,5,8 |
| 1586 | Sanguiin H6 | *Fa, Fv* | 3,10 |
| 1568 | Sanguiin H10 | *Fa, Fv* | 8,10 |

[a]Abbreviations: *Fa*, *F. x ananassa*, *Fc*, *F. chiloensis*, *Fv*, *F. vesca*

[b] Literature where the compound has been characterized by MS analysis. 1) Seeram *et al*. (2006); 2) Aaby *et al*. (2007); 3) Buendia *et al*. (2010); 4) Kajdzanoska *et al*. (2010); 5) Simirgiotis *et al*. (2010); 6) Cerezo *et al*. (2010); 7) Aaby *et al*. (2010); 8) Del Bubba *et al*. (2012); 9) Sun *et al*. (2014); 10) Gasperotti *et al*. (2013); 11) Josuttis *et al*. (2013).

ellagic acid and quercetin confirmed further fragmentation of the *m/z* 301 as ellagic acid derivatives with fragment ions at *m/z* 185 and *m*/z 145, and quercetin derivatives with ions at *m/z* 179 and *m/z* 151.

Quantification of the compounds was carried out using external standards. A calibration curve was made with pure compound, or a closely similar or basic unit of the compound when a pure reference standard was not available, at a range of concentrations, analyzed under the same conditions, and yielded linear regression coefficients greater than 0.990. The specific external standards used are indicated in each table.

Final values of the content of each compound were corrected for loss during extraction by calculating the total anthocyanin as the sum of each individual anthocyanin detected and the mean total anthocyanin from 3 replicate extractions of fruit of each genotype in 80% MeOH (Chapter 3). This ratio of total anthocyanins from each method was used a correction factor across all compounds.

### 4.2.5 Statistical analysis

Because the objective was to determine if each *F. vesca* had a unique set of polyphenolic contents and antioxidant activity, each cultivar was considered a unique genotype for statistical analyses. Thus, to evaluate significant differences among genotypes, analyses of variance (ANOVA) were performed (Sigmaplot for Windows, v. 12.0), and genotype means were compared using Fisher's LSD at P=0.05.

## 4.3 Results and Discussion

### 4.3.1 Phenolic compound identification

The phenolic compounds in *F. vesca* and *F. x ananassa* berries were classified using their chromatographic behavior and mass spectra (both MS and MS/MS) for comparison to spectra of available standards and/or those in the literature. The MS fragmentation patterns of only specific compounds found in this study were discussed. They are presented by classification into six groups: anthocyanins, flavonols, hydroxycinnamic acids, flavan-3-ols, and ellagitannins and ellagic acid derivatives, with the results summarized for targeted (with available standards) and tentatively-identified compounds in either positive or negative modes.

Anthocyanins. Anthocyanins are responsible for the red color of strawberries. Identification of anthocyanins (Figure 4.1A, Table 4.2) was conducted in the positive ion mode with the most abundant protonated parent ions $[M-H]^+$ at *m/z* 449 and *m/z* 433 (peaks 5 and 10, respectively) for the red *F. vesca* Baron Solemacher. A loss of neutral fragments with a mass of 162 Da provided ions at *m/z* 287 and 271, respectively, establishing the presence of cyanidin-3-glucoside and pelargonidin-3-glucoside. These were also confirmed by comparing retention times and mass fragmentation with authentic aglycone and derivative standards. Another pseudomolecular ion $[M-H]^+$ at *m/z* 463 (peak 17) yielded a daughter ion at *m/z* 301, indicating a loss of a hexose unit, and was tentatively identified as peonidin-3-glucoside.

Pelargonidin malonyl-glucoside (peak 25) showed a protonated parent ion $[M-H]^+$ at *m/z* 519 with consecutive fragmented ions at *m/z* 433 (a loss of a malonyl moiety, 86 Da) and at *m/z* 271 (a further loss of a glucose unit, 162 Da). Peonidin-malonyl-glucoside (peak 12) was tentatively identified with a parent ion $[M-H]^+$ at *m/z* 549 and dissociation ions at *m/z* 463 and 301. Finally, a cyanidin-malonyl-glucoside ion (peak 27) ($[M-H]^+$) at *m/z* 535 was tentatively identified from the presence of a major fragment at *m/z* 287 indicative of cyanidin aglycone. The parent peak fragmented to *m/z* 449 (a loss of a malonyl moiety, 86 Da) and *m/z* 287 (a subsequent loss of a hexose moiety, 162 Da).

The dominant anthocyanins in red *F. x ananassa, F. chiloensis* and *F. vesca* have included derivatives of pelargonidin-3-glucoside and cyanidin-3-glucoside (Kosar *et al*., 2004; Seeram *et al*., 2006; Aaby *et al*., 2007, 2012; Buendia *et al*., 2010; Kajdzanoska *et al*., 2010; Simirgiotis *et al*., 2010; Cerezo *et al*., 2010; Kelebek *et al*., 2011; Munoz *et al*., 2011; Del Bubba *et al*., 2012; Sun *et al*., 2014). Cyanidin and pelargonidin derivatives have also been reported from white *F. chiloensis* spp*. chiloensis* var. *chiloensis* (Cheel *et al.*, 2005; Simirgiotis *et al.,* 2010). In the present study, pelargonidin derivatives were not detected in two of the white *F. vesca*, Ivory and Yellow Wonder. Derivatives of peonidin were detected in the red and white mutants of all six cultivars of *F. vesca* and the two cultivars of *F. x ananassa*. Peonidin-malonyl glucoside was previously found in red and white *F. vesca (*Del Bubba *et al*., 2012; Sun *et al*., 2014), and peonidin-3-glucoside was recently reported in red *F. x ananassa* (Cerezo *et al*., 2010).

Figure 4.1

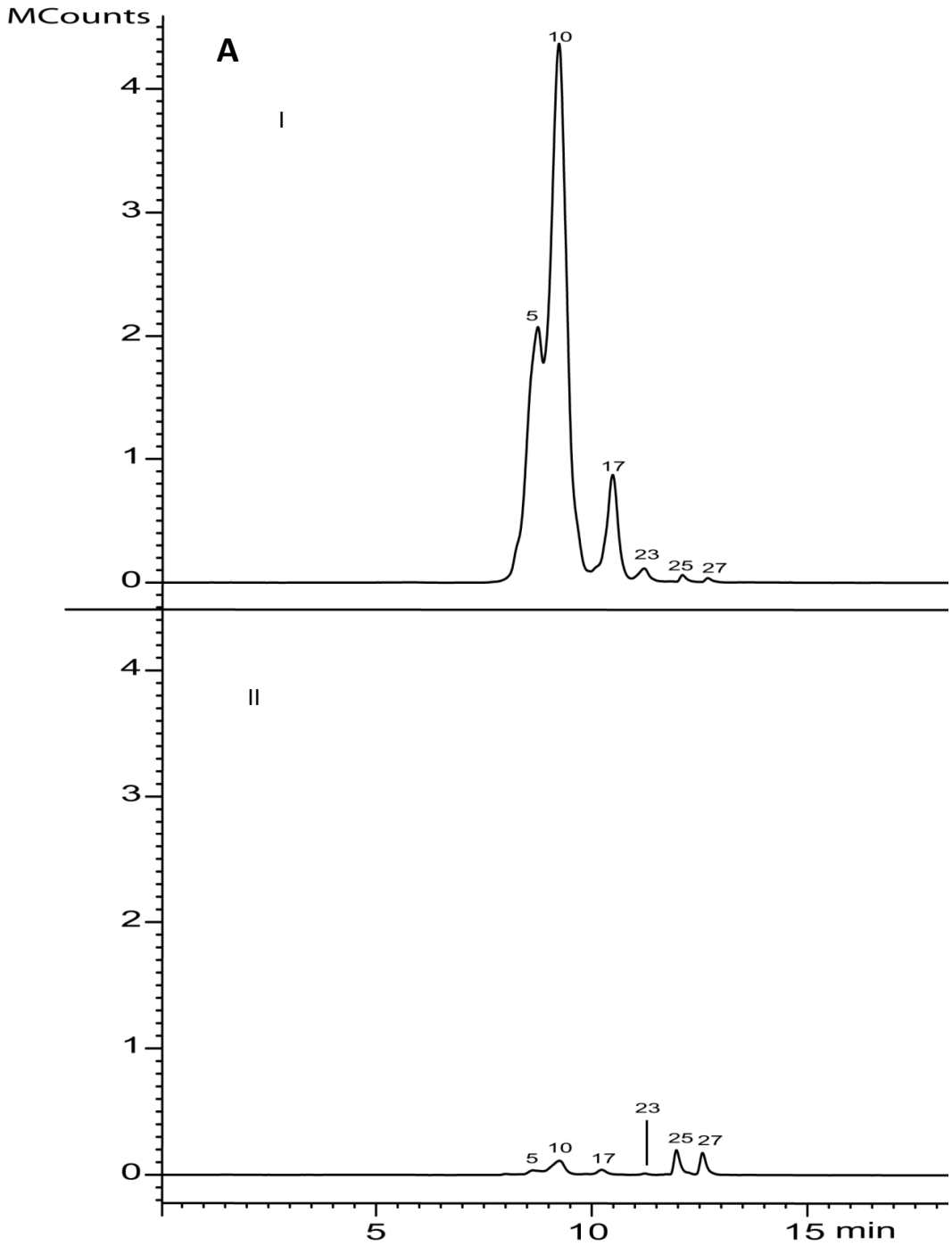


Figure 4.1 (Continued)

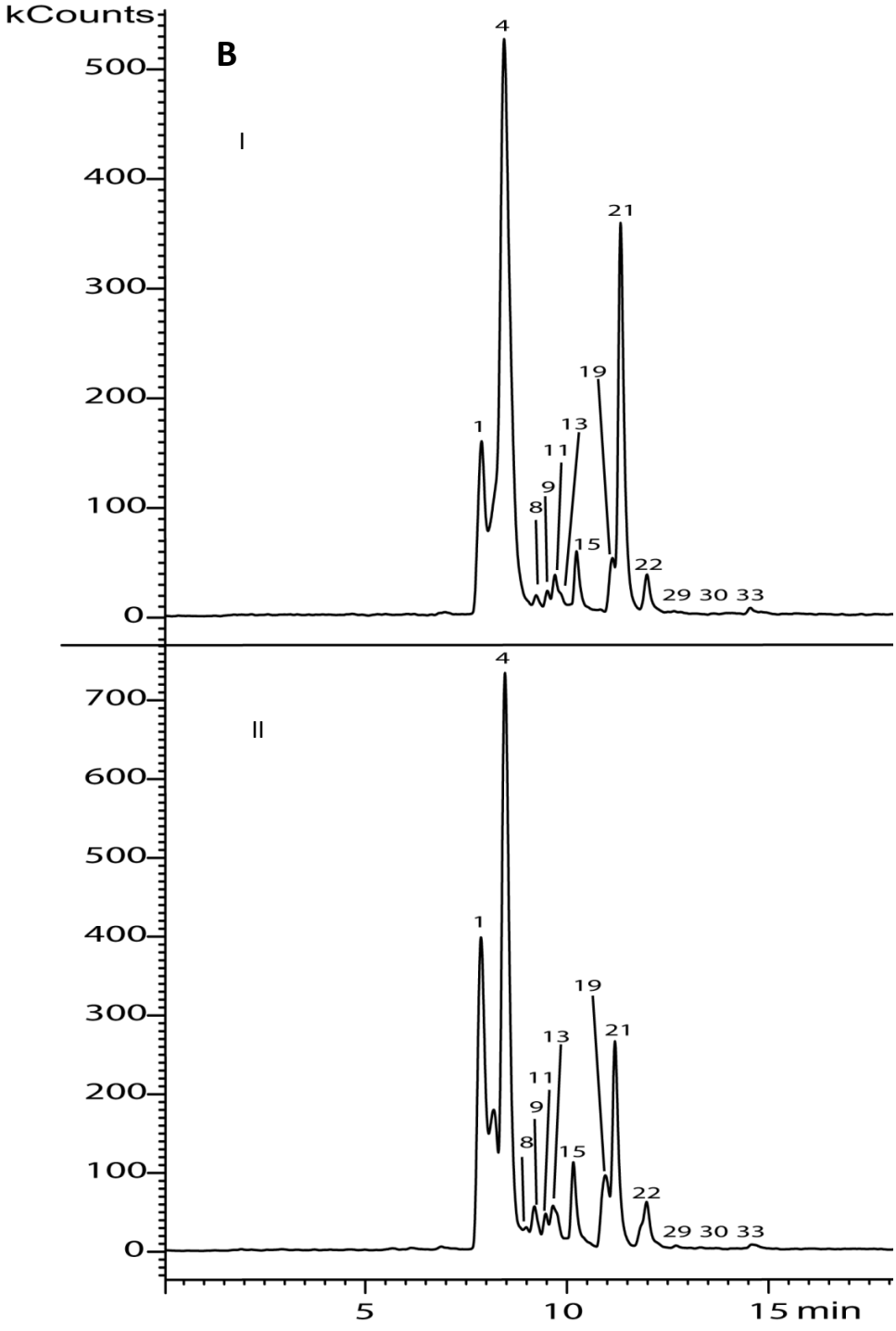


Figure 4.1 (Continued)

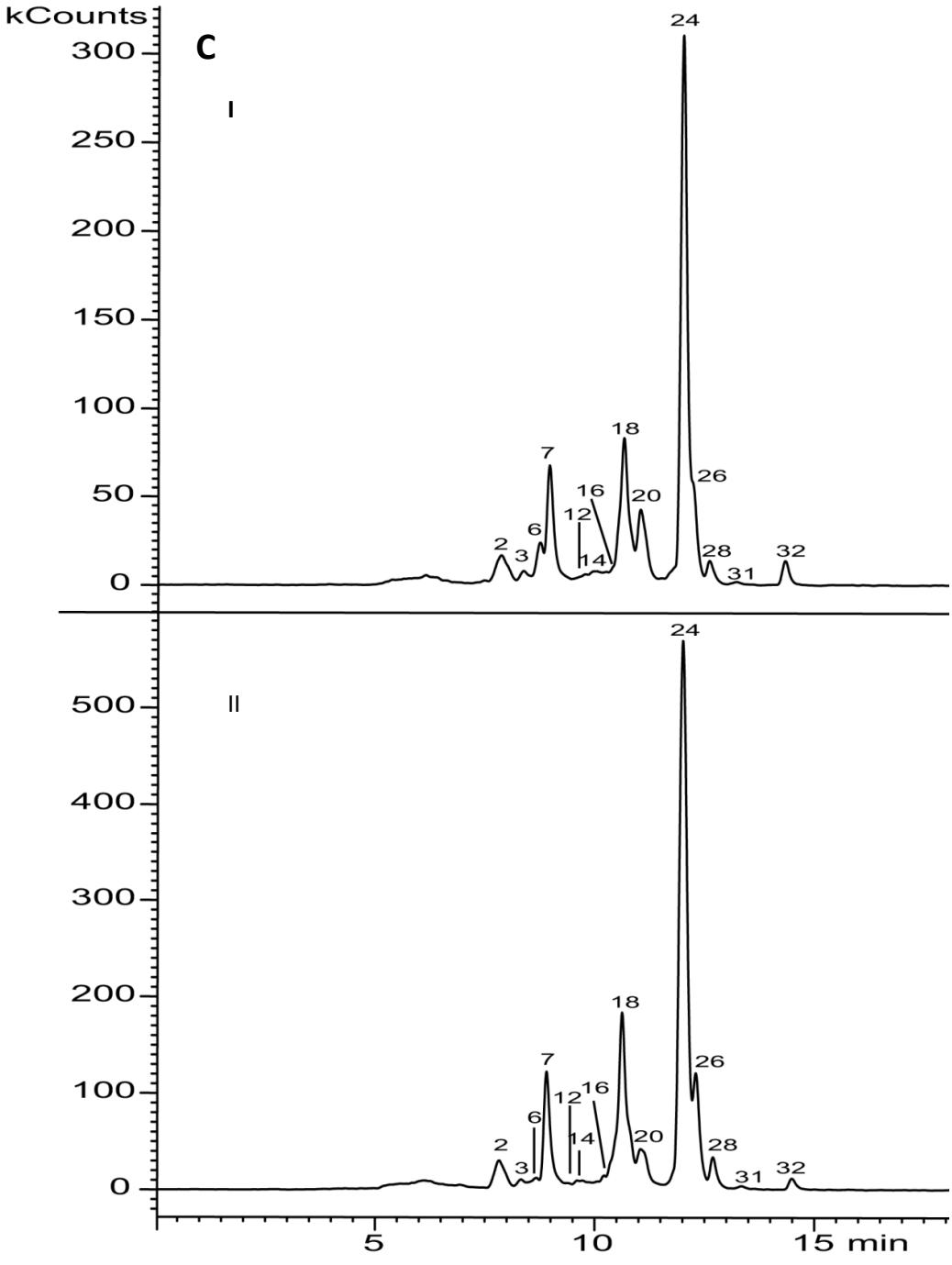



**Figure 4.1** HPLC-ESI-MS/MS extracted ion chromatograms of red (I) and white (II) *F. vesca* and *F. x ananassa* berries. A) Compounds in positive ion mode, peak numbers in Table 4.2; B) compounds with authentic standards in negative mode, peak numbers in Table 4.2; C) tentatively-identified compounds in negative ion mode, peak numbers in Table 4.2.

**Table 4.2** Tentative identification of phenolic compounds in strawberry fruit by HPLC=ESI-MS. Species-cultivars. (color) were: *Fragaria x ananassa:* Er=Earliglow (red), WPw=White Pine (white); *F. vesca*: BSr=Baron Solemacher (red), PCw=Pineapple Crush (white), and WSw=White Soul (white). All includes the white *F. vesca* cultivars Baron Solemacher and Ivory.

| Peak | Rt (min) | MW | MS(*m/z*) | MS/MS (*m/z*) | Tentative Identification | Cultivars |
|---|---|---|---|---|---|---|
| **1** | 7.2 | 578 | 577 $[M-H]^-$ | 425, 407, 289 | Proanthocyanidin dimer | All |
| **2** | 7.6 | 326 | 325 $[M-H]^-$ | 265,187,163,119 | *p*-Coumaroyl- hexose | All |
| **3** | 8.1 | 634 | 633$[M-H]^-$ | 463, 301, 185 | HHDP galloyl hexoside | All |
| **4** | 8.3 | 290 | 289 $[M-H]^-$ | 245, 203, 109 | (+) Catechin | All |
| **5** | 8.5 | 448 | 449$[M+H]^+$ | 287 | Cyanidin-3-glucoside | All |
| **6** | 8.6 | 1586 | 783 [M-2H]2-/2 | 935,633,613, 301 | Sanguiin H10 | All |
| **7** | 8.7 | 490 | 489[M-H]- | 447, 285, 255 | Kaempferol acetyl glucoside | All |
| **8** | 8.9 | 354 | 353$[M-H]^-$ | 191 | Cholorogenic acid | All |

**Table 4.2 (continued)**

| | | | | | | |
|---|---|---|---|---|---|---|
| **9** | 9.1 | 180 | 179[M-H]$^-$ | 135 | Caffeic acid | All |
| **10** | 9.2 | 432 | 433[M+H]$^+$ | 271 | Pelargonidin-3-glucoside | Er, WPw, BSr, PCw, WSw |
| **11** | 9.4 | 578 | 577 [M-H]$^-$ | 425, 407, 289 | Proanthocyanidin dimer | All |
| **12** | 9.5 | 434 | 433 [M-H]$^-$ | 301, 300 | Ellagic acid pentoside | All |
| **13** | 9.6 | 290 | 289 [M-H]$^-$ | 245, 203, 109 | (-) Epicatechin | All |
| **14** | 9.7 | 935 | 934[M-H]$^-$ | 633, 301, 257 | Galloyl bis HHDP hexose | All |
| **15** | 10.1 | 578 | 577 [M-H]$^-$ | 425, 407, 289 | Proanthocyanidin dimer | All |
| **16** | 10.2 | 326 | 325 [M-H]$^-$ | 265,187,163,119 | *p*-Coumaroyl-hexose | All |
| **17** | 10.3 | 462 | 463[M+H]$^+$ | 301 | Peonidin-3-glucoside | All |
| **18** | 10.4 | 434 | 433 [M-H]$^-$ | 301,300 | Ellagic acid pentoside | All |

**Table 4.2 (continued)**

**Table 4.2 (continued)**

| | | | | | | |
|---|---|---|---|---|---|---|
| **19** | 10.7 | 164 | 163 $[M-H]^-$ | 119 | *p*-Coumaric acid | All |
| **20** | 10.9 | 448 | 447$[M-H]^-$ | 301,300,257,185 | Ellagic acid deoxyhexoside | All |
| **21** | 11.2 | 302 | 301$[M-H]^-$ | 185, 145 | Ellagic acid | All |
| **22** | 11.7 | 464 | 463$[M-H]^-$ | 301, 179, 151 | Quercetin-3-glucoside | All |
| **23** | 11.8 | 518 | 519$[M+H]^+$ | 433, 271 | Pelargonidin-malonyl-glucoside | Er, WPw, BSr, PCw, WSw |
| **24** | 11.9 | 935 | 934$[M-H]^-$ | 633, 301, 257 | Galloyl bis HHDP hexose | All |
| **25** | 12 | 548 | 549$[M+H]^+$ | 463,301 | Peonidin-malonyl-glucoside | All |
| **26** | 12.3 | 448 | 447$[M-H]^-$ | 315,300,257,185 | Methyl ellagic acid pentoside | All |
| **27** | 12.5 | 534 | 535$[M+H]^+$ | 449,287 | Cyanidin-malonyl-glucoside | All |

**Table 4.2 (continued)**

| | | | | | | |
|---|---|---|---|---|---|---|
| **28** | 12.6 | 462 | 461[M-H]$^-$ | 446, 315, 301 | Dimethyl ellagic acid pentoside | All |
| **29** | 12.7 | 478 | 477 [M-H]$^-$ | 301, 179, 151 | Quercetin-3-glucuronide | All |
| **30** | 13.2 | 448 | 447 [M-H]$^-$ | 285, 255 | Kaempferol-3-glucoside | All |
| **31** | 13.3 | 490 | 489[M-H]$^-$ | 447, 285, 255 | Kaempferol acetyl glucoside | All |
| **32** | 14.4 | 594 | 593[M-H]$^-$ | 447, 285, 255 | Kaempferol coumaroyl hexoside | All |
| **33** | 14.5 | 302 | 301[M-H]$^-$ | 179, 151 | Quercetin | all |

Hydroxycinnamic acids. Derivatives of caffeic acid and *p*-coumaric acid have been the most common hydroxycinnamic acids detected in *F. x ananassa* and *F. vesca* (Määttä-Riihinen *et al*., 2004; Buendia *et al*., 2010; Kajzdzanoska *et al*., 2011; Munoz *et al*., 2011; Del Bubba *et al*., 2012; Sun *et al*., 2014). By comparison to authentic standards in negative HPLC-MS mode, chlorogenic and caffeic acid (peaks 8 and 9, Table 4.2) were confirmed in all of the genotypes studied in the present work. Chlorogenic and caffeic acid had $[M-H]^-$ ions at *m/z* 353 and 179 with fragment ions at 191 and 135, respectively. Peak 19 showed an ion at *m/z* 163 with a fragment ion at *m/z* 119 and was confirmed as *p*-coumaric acid with the same retention time as the authentic standard. However, *p*-coumaric acid was not detected in three of the white cultivars of *F. vesca. p*-Coumaroyl hexose (peaks 2 and 16, Table 4.2) showed the loss of a hexose residue as the monocharged pseudo-molecular $[M-H]^-$ ion at 325 exhibited a fragment ion at 163. This compound was reported by others (Seeram *et al*., 2006; Aaby *et al*., 2007, 2012; Kajzdnoska *et al*., 2010; Kelebek *et al*., 2011) in red *F. x ananassa,* and in *F. vesca (*Del Bubba *et al*., 2012; Sun *et al*., 2014), and was found here in both white and red cultivars of *F. x ananassa* and *F. vesca.*

Flavonols. Quercetin and kaempferol derivatives were the major flavonols detected in both red and white *F. vesca* and *F. x ananassa* berries (Fig. 4.1B, Table 4.2). Quercetin-3-glucoside (peak 22) showed a $[M-H]^-$ ion at *m/z* 463 with the dissociation ion at *m/z* 301 (a loss of a glucose unit) and characteristic fragment ions at *m/z* 179 and *m/z* 151. The identity was confirmed with an authentic standard. Quercetin-3-glucoronide (peak 29) was identified due to its $[M-H]^-$ ion at *m/z* 477 with a key fragment ion at *m/z* 301 (a loss of a glucoronic acid moiety, 176 Da) and further dissociation ions at *m/z* 179 and 151. The free form of quercetin (peak 33) was also identified with the same retention time and $[M-H]^-$ ion at *m/z* 301 with quercetin specific daughter ions at m/z 179 and 151 as with the authentic standard. Others also reported these compounds in *Fragaria* spp. (Table 4.1).

Kaempferol-3-glucoside (peak 30) showed a parent ion at *m/z* 447 producing a fragment at *m/z* 285 owing to the loss of a glucose moiety, and one at *m/z* 255 which is a characteristic fragment of kaempferol. The retention time and fragmentation pattern of an authentic standard confirmed the identity. Three additional peaks were identified as kaempferol derivatives as the $[M-H]^-$ produced ions of kaempferol aglycone at *m/z* 285. Kaempferol-acetyl glucoside (peaks 7 and 31, Fig. 4.1B, Table 4.2) showed a pseudo-molecular ion $[M-H]^-$ at *m/z* 489 with fragments at *m/z* 447 (a loss of an acetylglucose

group, 204 Da) and at *m/z* 255, as has been identified in *F. x ananassa* (Buendia *et al.,* 2010; Kajdzanoska *et al*., 2010) but not in *F. chiloensis*. On the basis of the fragmentation pattern reported by others (Seeram *et al*., 2006; Aaby *et al*., 2007, 2012; Kajzdnoska *et al*., 2010; Simirgiotis *et al*., 2010), kaempferol-coumaroyl hexoside (peak 32) was tentatively identified. This late-eluting kaempferol glycoside showed a $[M-H]^-$ ion at *m/z* 593 with subsequent MS/MS ions at *m/z* 447 (loss of a coumaroyl residue, 146 Da) and at *m/z* 285 (further loss of a hexose residue, 161 Da). Simirgiotis *et al*. (2010) reported kaempferol coumaroyl hexoside in white *F. chiloensis*, and kaempferol acetyl glucoside was found in red and white *F. vesca* (Del Bubba *et al.,* 2012; Sun *et al*., 2014).

Flavan-3-ols. Proanthocyanidins are oligomers or polymers of flavan-3-ols and are also known as condensed tannins. The common flavan-3-ols in strawberry are derived from catechin and epicatechin. Only homogenous B-type proanthocyanidin has been reported in strawberry (Gu *et al*., 2003). Proanthocyanidin monomers were identified based on their mass spectra and by comparison with the retention time of authentic external standards. Catechin and epicatechin (peaks 4 and 13, Table 4.2) showed a deprotonated molecular ion $[M-H]^-$ at *m/z* 289 with MS/MS ions at *m/z* 203 and *m/z* 109. These isomers were distinguished by comparison with retention times of the external standards. Peaks 1, 11 and 15 showed the same $[M-H]^-$ ion at *m/z* 577, and the same fragments at *m/z* 451 (loss of 126 Da), 425 (characteristic fragment by retro Diels-Alder reaction) (Gu *et al*., 2003), and 407 and 289 (corresponding to the loss of a catechin or epicatechin unit). Based on the fragmentation pattern, these peaks can be identified as proanthocyanidin dimers (Buendia *et al*., 2010; Kajdzanoska *et al*., 2011; Aaby *et al*., 2012; Del Bubba *et al*., 2012; Sun *et al*., 2014). Other dimers, trimers, tetramers and pentamers were not detected in contrast to other studies of *F. x ananassa* and *F. vesca* (Seeram *et al*., 2006; Buendia *et al*., 2010; Kajzdzanoska *et al*., 2011; Del Bubba *et al*., 2012; Josuttis *et al*., 2013; Sun *et al*., 2014) .

Ellagic acid forms. Free ellagic acid and ellagic acid conjugates were abundantly present in the genotypes in this study. Free ellagic acid (peak 21, Figure 4.2C, Table 4.2) was confirmed as it had a $[M-H]^-$ at *m/z* 301, and a retention time and characteristic fragment ions at *m/z* 257 and 185 matching an authentic standard. Ellagic acid pentoside (peaks 12 and 18, Table 4.2) showed a $[M-H]^-$ ion at *m/z* 433 with fragment ions at *m/z* 301 (a loss of a pentose moiety, 132 Da), 300 and 257. Ellagic acid deoxyhexoside (peak 20) was tentatively identified with a $[M-H]^-$ ion at *m/z* 447 and MS/MS ions at *m/z* 301 (a loss of a

deoxyhexoside unit, 146 Da), 300, 257 and 185. Peaks 12, 18 and 20 showed MS/MS ions at 257 and 185 which are specific ions for ellagic acid. This fragmentation pattern was also assigned to ellagic acid deoxyhexoside in *F. x ananassa* (Aaby *et al*., 2007, 2012; Kajzdnoska *et al*., 2010; Gasperotti *et al*., 2013; Sun *et al*., 2014). Methyl ellagic acid pentoside (peak 26) showed a $[M-H]^-$ ion at *m/z* 447 with a loss of a pentoside residue (132 Da) and a methyl group during fragmentation to *m/z* 315 and *m/z* 300, respectively. Dimethyl ellagic acid pentoside was identified as peak 28 which had a $[M-H]^-$ ion at *m/z* 461 and produced further MS/MS fragmentation to yield ions at *m/z* 461 and 446 (a loss of a methyl radical), 315 (a loss of a pentose), and 301 (a further loss of a methyl, leading to formation of free ellagic acid). The present data confirms the presence of the methyl ellagic glycosides in *F. vesca* reported by others (Del Bubba *et al*., 2012; Sun *et al*., 2014).

Ellagitannins are polymers of hexahydroxydiphenic acid (HHDP), which are dimeric forms of gallic acid. Ellagitannins show structural variability with linkage of HHDP residues with the glucose moiety (Fraser *et al*., 2011) Hydrolytic release of HHDP units from ellagitannins gives rise to bislactone ellagic acid. Gallic or HHDP acids may be esterified to form polymers which can be gallotannins or ellagitannins. HHDP-galloylhexose (peak 3, Table 4.2) had a $[M-H]^-$ ion at *m/z* 633 with MS/MS fragment ions at *m/z* 481 (a loss of a galloyl unit, 152 Da), 463 (a loss of a gallic acid, 170 Da), and 301 with principal ions at 257 and 185 for ellagic acid, as reported from cultivars of red *F. x ananassa* (Aaby *et al*., 2007; Kajdznoska *et al*., 2010; Josuttis *et al*., 2013) and *F. vesca* (Del Bubba *et al*., 2012; Gasperotti *et al*., 2013; Sun *et al*., 2014). Peak 6 (Table 4.2) was identified as sanguiin H10 as it showed a $[M-2H]^{2-}/2$ ion at *m/z* 935, with fragment ions at 633, 613 and 301*(*Del Bubba *et al*., 2012; Gasperotti *et al*., 2013; Sun *et al*., 2014). Galloyl-bis-HHDP hexose (peak 14 and 24) also known as casuarictin/potentillin (Aaby *et al*., 2007; Del Bubba *et al*., 2012; Gasperotti *et al*., 2013) was tentatively identified with the main product ions at *m/z* 935 and MS/MS ions at 633 *(*a loss of HHDP moiety, 302Da) and 301 (loss of HHDP-glucose, 634 Da) (Aaby *et al*., 2007, 2012; Del Bubba *et al*., 2012; Gasperotti *et al*., 2013). Close structural similarity and lack of commercially available standards make it difficult to identify specific ellagitannins with HPLC-MS. The major ellagitannin argimoniin, a dimer of galloyl bis-HHDP glucose (Aaby *et al*., 2007, 2012; Gasperotti *et al*., 2013), and lambertenin C (Gasperotti *et al*., 2013), were reported in strawberry though they were not found in this study or other studies of *F. vesca* (Del Bubba *et al*., 2012; Sun *et al.,* 2014). Del Bubba *et al*. (2012) and Gasperotti *et al*. (2013) reported the presence of castalagin in *F. vesca,* but it was not detected in either *F.*

*vesca* or *F. x ananassa* in the present study or the red or white *F. vesca* studied by Sun *et al.* (2014).

### 4.3.2 Polyphenol content

As might be expected of red versus white fruit, there were significant differences in anthocyanin content between the red and white cultivars within *F. vesca* and *F. x ananassa* (Table 4.3), similar to that reported in a comparison of red versus white cultivars of *F. chiloensis* (Simirgiotis *et al*., 2010). The total anthocyanin content of white *F. vesca* berries Pelargonidin-3-glucoside was the major anthocyanin in red *F. x ananassa* cv. Earliglow followed by cyanidin-3-glucoside. Anthocyanin content of the red *F. x ananassa* in the present study showed values similar to those reported in some studies *(*Kosar *et al*., 2004; Aaby *et al*., 2012; Kelebek *et al*., 2010), although lower than other reports (Määttä-Riihinen *et al*., 2004; Buendia *et al*., 2010; Simirgiotis *et al*., 2010; Cerezo *et al*., 2010). The content of pelargonidin-3-malonylglucoside and cyanidin-3-malonylglucoside in *F. x ananassa* were similar to values reported by Aaby *et al*. (2012), but content of the former compound was higher than reported by others (Määttä-Riihinen *et al*., 2004; Kelebek *et al*., 2011). Cyanidin-3-glucoside was the primary anthocyanin in red *F. vesca*, and the cyanidin derivatives were the only anthocyanins detected in 3 of 5 white *F. vesca*. In contrast, Simirgiotis *et al*. (2010) reported pelargonidin-3-glucoside as the major anthocyanin in a red *F. chiloensis* and cyanidin-3-glucoside as the dominant anthocyanin in a white *F. chiloensis*. The white cultivar of *F. x ananassa* showed five-fold higher values for cyanidin-3-malonylglucoside than the white cultivars of *F. vesca.*

Total flavonol content was lower in red and white cultivars of *F. vesca* (ranging from 0.8 to 1.9 mg/100 g FW) and the white *F. x ananassa* (2.9 mg/100 g FW) than the red *F. x ananassa* (8.2 mg/100 g FW) (Table 4.4). However, within *F. vesca*, total flavonol levels of red and three of the white cultivars were comparable. Only Pineapple Crush and White Soul total flavonol content were significantly lower, differences also observed for some of the individual flavonol compounds as well. Quantitatively, quercetin-3-glucoronide was the predominant flavonol across all cultivars, comprising 37 to 60% of the total in *F. vesca* and over 70% in *F. x ananassa*. Quercetin-3-glucoronide was also the predominant flavonol in red *F. x ananassa* (Buendia *et al*., 2010; Kelebek *et al*., 2011; Aaby *et al*., 2012), at levels comparable to Earliglow in the present work.

**Table 4.3** Individual and total anthocyanin content (mg/100g of fresh weight) of cultivars from *Fragaria vesca* and *Fragaria* x *ananassa*.

| Cultivar | Color | Pg-3-glu[z] | Pg-mal glu[y] | Cy-3-glu | Cy-mal glu[x] | Total |
|---|---|---|---|---|---|---|
| | | | *Fragaria vesca* | | | |
| Baron Solemacher | Red | 14.1 ± 0.2[w] b[v] | 2.1 ± 0.9 b | 20.2 ± 1.1 a | 2.7 ± 0.1 b | 39.1 ± 1.5b |
| Baron Solemacher | White | 0.1 ± 0 c | nd[u] | 0.6 ± 0 b | 0.7 ± 0.1 c | 1.3 ± 0.6 cd |
| Yellow Wonder | White | nd | Nd | 0.6 ± 0 b | 0.3 ± 0 d | 0.8 ± 0.1 d |
| Pineapple Crush | White | nd | Nd | 0.4 ± 0 b | 0.6 ± 0 d | 1.0 ± 0 .4d |
| Ivory | White | nd | Nd | 0.3 ± 0 b | 0.2 ± 0 d | 0.5 ± 0 d |
| White Soul | White | 0.1 ± 0 c | 0.1 ± 0 c | 0.6 ± 0 b | 0.8 ± 0 c | 1.6 ± 0.1 cd |
| | | | *Fragaria x ananassa* | | | |
| Earliglow | Red | 29.2 ± 1 a | 3.9 ± 0.2 a | 21.0 ± 1.2a | 3.1 ± 0.1[u] a | 71.0 ± 2.3 a |
| White Pine | White | 0.1 ± 0 c | 0.2 ± 0 c | 0.7 ± 0 b | 3.1 ± 0.1a | 4.2 ± 0.1 c |

Table 4.3 (continued)

[z]Abbreviations: Pg =pelargonidin; cy=cyanidin; Peo=peonidin; glu=glucoside; mal=malonyl

[y] Quantified as mg of pelargonidin-3-glucoside/100 g FW

[x]Quantified as mg of cyanidin-3-glucoside/100 g FW.

[w]Means (*n=3*) ± SD. SD=0 if ≤0.5 mg/100 g FW.

[v]Means in the same column across species followed by different letters are significantly different by Fisher's LSD at $P<0.05$.

[u]nd indicates it was not detected.

**Table 4.4** Individual and total flavonol content (mg x $10^3$ /100g fresh weight) of cultivars from *Fragaria vesca* and *Fragaria* x *ananassa*.

| Cultivar | Color | K-3-gluc[z] | K-3-act-glu[y] | K-cou-hex[y] | Quercetin | Q-3-β-D-glu | Q-3-glr | Total |
|---|---|---|---|---|---|---|---|---|
| | | | | *Fragaria vesca* | | | | |
| Baron Solemacher | Red | 111 ± 6[x] bc[w] | 142 ± 3 ns | 56 ± 4 c | 142 ± 4 ab | 557 ± 14 b | 590 ± 16 de | 1599 ± 10 c |
| Baron Solemacher | White | 82 ± 3 cd | 171 ± 0 | 122 ± 12 abc | 55 ± 1 cd | 306 ± 7 c | 739 ± 14 cd | 1476 ± 28 cd |
| Yellow Wonder | White | 137 ± 12 b | 120 ± 0 | 175 ± 46 a | 137 ± 2 abc | 259 ± 4 d | 984 ± 10 c | 1818 ± 34 c |
| Pineapple Crush | White | 82 ± 3 cd | 10 ± 0 | 34 ± 0 c | 85 ± 6 bcd | 162 ± 7 f | 424 ± 19 de | 790 ± 45 d |
| Ivory | White | 72 ± 5 d | 51 ± 6 | 167 ± 55 ab | 210 ± 32 a | 222 ± 2 e | 904 ± 46 c | 1619 ± 79 c |

Table 4.4 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| White Soul | White | 85 ± 3 cd | 161 ± 9 | 157 ± 13 ab | 14 ± 1 d | 54 ± 0 h | 255 ± 7 e | 720 ± 32 d |
| | | | | *Fragaria x ananassa* | | | | |
| Earliglow | Red | 871 ± 20 a | 681 ± 20 | 68 ± 20 c | 55 ± 5 cd | 729 ± 16 a | 5835 ± 284 a | 8238 ± 1181 a |
| White Pine | White | 133 ± 1 b | 329 ± 4 | 82 ± 0 bc | 30 ± 0 d | 86 ± 4 g | 2281 ± 44 b | 2998 ± 45 b |

[z]Abbreviations: K = kaempferol; Q = quercetin; glu = glucoside; glr = glucoronide; act = acetyl; hex = hexoside; cou = coumaroyl.

[y] Quantified as mg of kaempferol-3-glucoside/100 g FW.

[x]Means (*n=3*) ± SD, except for kaempferol-3-acetyl glucoside and kaempferol-coumaroyl hexoside for which *n*=2. Total flavonol was also calculated from 2 complete replications. SD=0 if ≤0.5 mg/100 g fresh weight.

[w]Means in the same column across species followed by different letters are significantly different by Fisher's LSD at $P<0.05$. ns indicates no significant difference among means.

varied from 0.1 to 1.6 mg/100 g FW, which was 28-fold less than the red *F. vesca*. The white *F. x ananassa* had nearly 17-fold less total anthocyanin than the red cultivar.

The flavan-3-ols (proanthocyanidins) were one of the most abundant flavonoid groups across all *Fragaria* genotypes (Table 4.5). Proanthocyanidin dimers were the predominant flavan-3-ol, followed by catechin and very low levels of epicatechin. The red cultivars had higher levels of proanthocyanidin dimers than the white cultivars within the respective species, though no patterns were evident for catechin or epicatechin. The content of proanthocyanidin dimers and catechin in *F. x ananassa* cv. Earliglow was higher than the values reported by Aaby *et al*. (2012), but lower than the levels found by others (Buendia *et al*., 2010; Josuttis *et al*., 2013). There was a significant difference in total proanthocyanidin content among white cultivars of *F. vesca* with Ivory showing the highest content and White Soul the lowest content.

Overall, the free hydroxycinnamic acid content was very low (<1% of total phenolics) with values varying from 0.07 to 2.8 mg/100 g FW (Table 4.6). This is 10-fold lower than values reported by others (Buendia *et al*., 2010; Kelebek *et al*., 2011) for red *F. x ananassa*. *p*-Coumaroyl hexose content was considerably higher in both red *F. x ananassa* and *F. vesca*, with Earliglow comparable to other red *F. x ananassa* (Buendia *et al*., 2010). Total hydroxycinnamic acid and chlorogenic acid content were highest in the white *F. vesca* White Soul followed by Ivory than in the other white *F. vesca* genotypes as well as the red genotype. This shift is similar to that observed in a white versus a red *F. chiloensis* genotype (Cheel *et al.,* 2007)

Ellagic acid content was higher in white *F. vesca* than the red cultivar of either species, but it was lowest (> 3-fold lower than white *F. vesca*) in the red and white *F. x ananassa* (Table 4.7). The free ellagic acid content was higher than the values reported for red *F. x ananassa* (Kosar *et al*., 2004; Aaby *et al*., 2012) and red and white *F. vesca* (Gasperotti *et al*., 2013). Three ellagic acid conjugates were quantified in the present study. EA pentoside content was higher in *F. x ananassa* than *F. vesca*, and the content in the white form was greater than the red form of the former species. In addition, there was variation among *F. vesca*, though not related to color mutation. Although methyl EA pentoside was lowest in red *F. x ananassa*, there were no clear differences between species or among color mutants of *F. vesca*. EA deoxyhexoside content was highest in red *F. x ananassa,* nearly 7-

**Table 4.5** Individual and total flavan-3-ols content (mg/100g fresh weight of cultivars of *Fragaria vesca* and *Fragaria* x *ananassa*.

| Cultivar | Color | Catechin | Epicatechin | PCD[zv] | Total |
|---|---|---|---|---|---|
| | | | *Fragaria vesca* | | |
| Baron Solemacher | Red | 9.2 ± 0[y] b[x] | 1.68 ± 1.2 ns | 127.3 ± 4.7 a | 138.5 ± 6 a |
| Baron Solemacher | White | 8.5 ± 0 b | 0.22 ± 0 | 38.3 ± 0.4 d | 47.5 ± 0.4 d |
| Yellow Wonder | White | 7.4 ± 0.1 c | 0.31 ± 0 | 50.8 ± 1.4 c | 58.5 ± 1.6 c |
| Pineapple Crush | White | 9.2 ± 0 b | 0.30 ± 0 | 37.0 ± 0.6d | 46.5 ± 0.6d |
| Ivory | White | 6.8 ± 0 c | 0.24 ± 0 | 61.8 ± 1.7 b | 68.8 ± 1.6 b |
| White Soul | White | 5.9 ± 0 d | 0.16 ± 0 | 18.2± 1.7 e | 24.3 ± 1.8 e |
| | | | *Fragaria x ananassa* | | |
| Earliglow | Red | 8.8 ± 0 b[y] | 0.18 ± 0.1 | 49.7 ± 0.4 c | 58.8 ± 0.4 c |
| White Pine | White | 12.8 ± 0 a | 0.25 ± 0 | 36.6 ± 1 d | 49.7± 1.5 d |

[z] proanthocyanidin dimers are quantified as mg of catechin/100 g FW.

[v]Abbreviation: PCD, Proanthocyanidin dimer [y] Means (*n=3*) ± SD. If SD≤0.05 mg/100 g fresh weight for (+) catechin, or ≤0.005 mg/100 g fresh weight for (-) epicatechin, then SD = 0. [x] Means in the same column across species followed by different letters are significantly different by Fisher's LSD at P=0.05.

**Table 4.6** Individual and total hydroxycinnamic acid content (mg x $10^3$/100g fresh weight of cultivars from *Fragaria vesca* and *Fragaria* x *ananassa*.

| Cultivar | Color | Caffeic acid | Chlorogenic acid | *p*-Coumaric acid | *p*-Coumaroyl hexose[z] | Total acids |
|---|---|---|---|---|---|---|
| | | | *Fragaria vesca* | | | |
| Baron Solemacher | Red | 6.0 ± 0.4[y] b[x] | 10.6 ± 0.3 e | 43.5 ± 0.1 b | 167 ± 4 b | 227 ± 5 d |
| Baron Solemacher | White | 10.1 ± 0.1 b | 16.5 ± 0.3 de | 10.3 ± 0.1 c | 39 ± 3 c | 75 ± 2 e |
| Yellow Wonder | White | 13.4 ± 0.1 b | 9.8 ± 0.1 e | 11.8 ± 0.2 c | 50 ± 5 c | 87 ± 4 e |
| Pineapple Crush | White | 0.8 ± 0.1 b | 23.8 ± 0.7 d | nd[w] | 43 ± 1 c | 69 ± 1 e |
| Ivory | White | 53.6 ± 11.5 a | 148.1 ± 6.8 c | Nd | 35 ± 1 c | 235 ± 10 c |
| White Soul | White | 15.9 ± 0.2 b | 441.3 ± 4.9 a | Nd | 50 ± 4 c | 506 ± 0 b |

Table 4.6 (continued)

*Fragaria x ananassa*

| | | | | | | |
|---|---|---|---|---|---|---|
| Earliglow | Red | 6.1 ± 0.1 b | 258.0 ± 5 b | 123.2 ± 5.2 a | 2427 ± 20 a | 2805 ± 14 a |
| White Pine | White | 8.0 ± 0.5 b | 15.3 ± 0.1 de | 3.1 ± 0 d | 27 ± 0.2 c | 54 ± 1 e |



[z]Quantified as mg of *p*-coumaric acid/100 g FW.

[y]Means (*n=3*) ± SD. SD=0 if ≤0.5 mg/100 g FW. Except *p*-coumaroyl hexose for which n=2. Total acids were calculated from 2 complete replications. SD = 0 if SD≤0.05 mg/100 g fresh weight. SD = 0 if SD≤0.05 mg/100 g fresh weight.

[x]Means in the same column across species followed by different letters are significantly different by Fisher's LSD at P<0.05.

[w]nd indicates it was not detected.

**Table 4.7** Free and conjugated ellagic acid (EA) content (mg/100g fresh weight) of cultivars from *Fragaria vesca* and *Fragaria* x *ananassa*.

| Cultivar | Color | EA | EAP[z] | MEAP[z] | EADH[z] | Total EA | GHH [z] | HGH [z] |
|---|---|---|---|---|---|---|---|---|
| | | | | *Fragaria vesca* | | | | |
| Baron Solemacher | Red | 13.4 ± 0.2[y] f[x] | 5.4 ± 0.6 e | 15.4 ± 1.5 a | 16 ± 1 e | 51 ± 1d | 29 ± 2 b | 18.3 ± 0.7b |
| Baron Solemacher | White | 41.0 ± 1.9 a | 6.7± 0.5 de | 9.5 ± 0.3 b | 50 ± 2b | 106 ± 5 b | 17 ± 1 c | 26.4 ± 0.9 a |
| Yellow Wonder | White | 25.7 ± 0.4 c | 6.5 ± 0.3de | 14.1 ± 0.2 a | 51 ± 1b | 98 ± 1 b | 11 ± 1 d | 26.4 ± 0.8 a |
| Pineapple Crush | White | 22.0 ± 0.7 d | 9.0 ± 0.1cd | 8.7 ± 0.2 b | 32 ± 0 c | 72 ± 1 c | 14 ± 1cd | 19.8 ± 1.4 b |
| Ivory | White | 17.1 ± 0.4 e | 5.5 ± 0.2 e | 7.7 ± 0 b | 25 ± 1 d | 55 ± 1d | 14 ± 3 cd | 10.9 ± 1.9 c |
| White Soul | White | 26.5 ± 0.2 bc | 10.0 ± 0.2 c | 14.0 ± 0.5 a | 46 ± 4 b | 97 ± 1 b | 24 ± 1 b | 25.0 ± 0.8a |
| | | | | *Fragaria x ananassa* | | | | |
| Earliglow | Red | 5.8 ± 0.6 gh | 16.0 ± 2 b | 1.4 ± 0.1 c | 114 ± 4a | 138 ± 6 a | 38 ± 1a | 6.1 ± 0.3d |

Table 4.7 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| White Pine | White | 4.5 ± 0.2 h | 37.4 ± 0.8a | 8.8 ± 0.3 b | 17± 2e | 67 ± 1 b | 11 ± 1 d | 6.9 ±1.1 d |

[z]Quantified as mg of ellagic acid/100 g FW.

[y] Means (*n=3*) ± SD, except for EA pentoside, methyl EA pentoside, EA deoxyhexoside, galloyl bis HHDP hexose and HHDP galloyl hexose for which *n*=2. Total flavonol was also calculated from 2 complete replications. SD = 0 if SD≤0.05 mg/100 g fresh weight.

[x]Means in the same column across species followed by different letters are significantly different by Fisher's LSD at P<0.05.

fold more than in white *F. x ananassa*. However, in contrast, the white forms of *F. vesca* had more EA deoxyhexoside than the red form. The total content of conjugates of EA reflected the EA deoxyhexoside differences as that pool contributed the major proportion to the totalfor most of the genotypes studied. In the present work, four EA forms were identified and quantified in *F. vesca*, one of which, methyl EA pentoside, was not reported by Gasperotti *et al*. (2013) but was identified by others (Sun *et al*., 2002; Del Bubba et a., 2012). Two ellagitannins, galloyl bis HHDP hexose and HHDP galloyl hexose, were also quantified in this study (Table 4.6). Galloyl bis HHDP hexose showed higher values in the present work for red *F. x ananassa* than prior reports (Buendia *et al*., 2010; Kelebek *et al*., 2011). HHDP galloyl hexose was greater in *F. vesca* than *F. x ananassa* although color-related differences were not evident. Gasperotti *et al*. (2013) identified 23 ellagitannins in red *F. x ananassa* and red and white *F. vesca,* but not the two reported here. However, both galloyl bis HHDP hexose and HHDP galloyl hexose have been found in *F. vesca* by others (Sun *et al*., 2002; Del Bubba *et al*., 2012), though levels were not reported. The reasons for these disparities are unknown, though they could be attributed to genotypic differences, environmental/production effects, or extraction techniques as noted above.

The non-anthocyanin (nonACY) flavonoid and ellagic acid/ellagitannin (EA/ET) pools reflect two major directions for carbon flux out of the shikimate pathway and into secondary metabolites. The present values are not broadly quantitative, but were hypothesized to represent relative values for each group to use for detecting changes in metabolic flux between species and color mutant genotypes. The total nonACY flavonoid content (total flavonols + total free and conjugated hydroxycinnamic acids + total flavan-3-ols) was significantly reduced in most white genotypes compared to the red within species (Fig. 4.2A). Among the white F. vesca, Ivory had the most and White Soul the least nonACY flavonoid content. Within *F. x ananassa*, total EA/ET content was lower in the white than the red cultivar. In contrast, in *F. vesca* the total EA/ET content was greater in three white genotypes compared to the red genotype, the content in two white genotypes were equal to the red, and the content in one white was less than the red (Fig. 4.2B). The total phenolic compound content, the sum of the nonACY flavonoids and EA/ETs, was similar to the differences in nonACY flavonoids alone in that the red genotypes had more than the white within species (Fig. 4.2C). Increased EA/ET content in three of the white *F. vesca* genotypes was not enough to compensate for the reduced levels of nonACY flavonoids, although it may indicate a shift towards the EA/ET pool nonetheless. The ratio of the nonACY flavonoids to

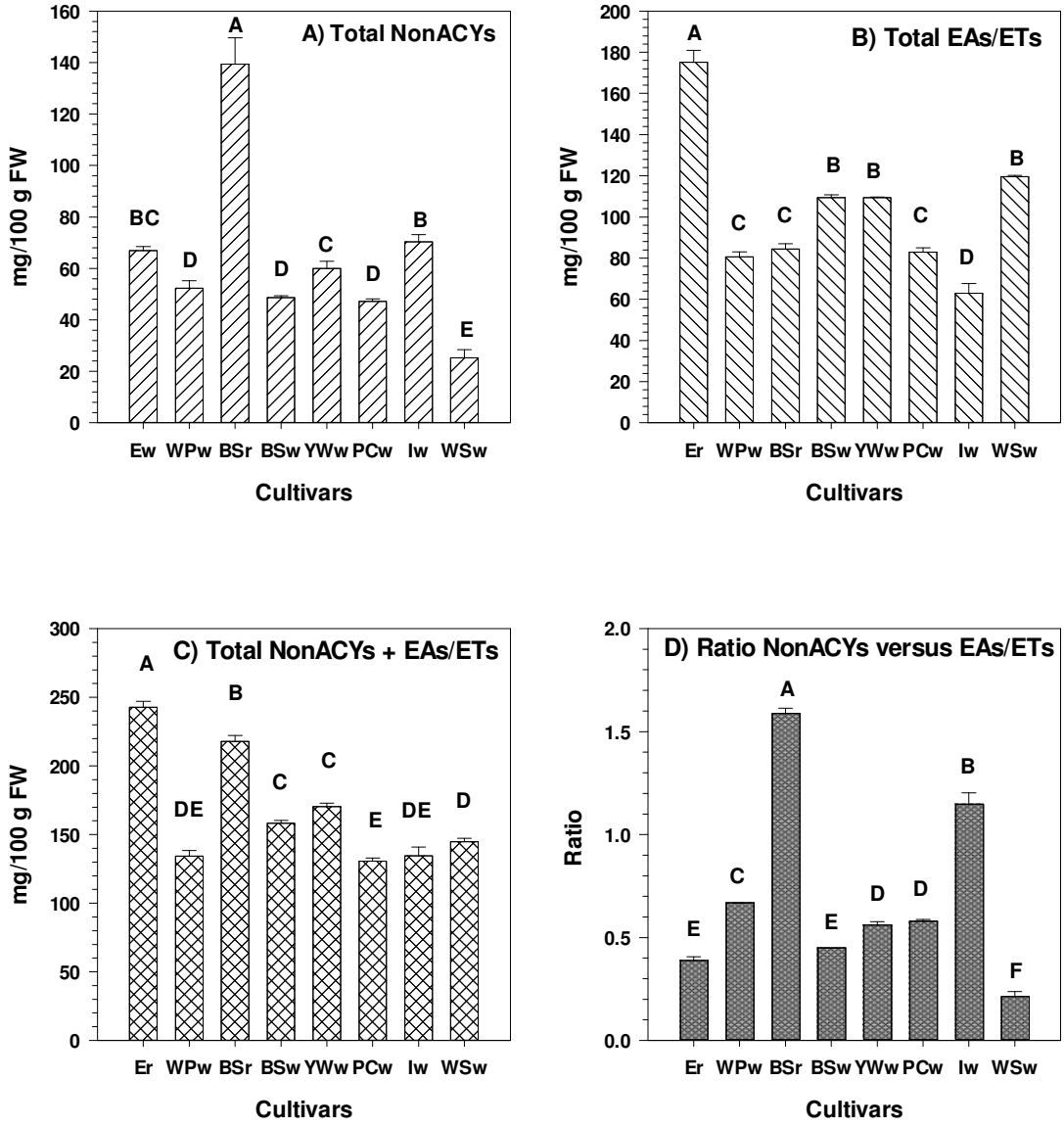



**Figure 4.2** (A) Total non-anthocyanin phenolic compound (NonACY) content(total flavonols + total free and conjugated hydroxycinnamic acids + total flavan-3-ols)B) total ellagic acid/ellagitannin-derived compound (EA/ETs) content, C) total NonACY + EA/ETs, and D) ratio of nonACY to EA/ETs**.** Er=Earliglow (red), WPw=White Pine (white); *F. vesca*: BSr=Baron Solemacher (red), BSw = Baron Solemacher (white), YWw=Yellow Wonder (white), PCw=Pineapple Crush (white), Iw=Ivory (white), and WSw=White Soul (white). Totals and ratios (*n*=2 or 3) below different letters are significantly different by Fisher's LSD at P=0.05.

EA/ETs was less than 1.0 for the red *F. x ananassa* but was greater than 1.5 for the red *F. vesca* (Fig. 4.2D). The ratio of the white *F. x ananassa* was significantly greater than the red, but the white *F. vesca* were all significantly lower than the red *F. vesca*. Within the white *F. vesca*, the ratio of one genotype Ivory remained above 1.0 while all others were less than 0.6.

The plant shikimate pathway produces metabolites which can enter the phenylpropanoid (Fraser *et al*., 2011) and ellagitannin (Niemetz *et al*., 2005) biosynthetic pathwaays and generate an enormous array of important secondary metabolites. Ellagic acid and ellagitannins are derived from gallic acid, the product of dehydrogenation of dehydroshikimic acid, whereas anthocyanins, flavonols and proanthocyanidins are derived from phenylalanine, a product much further downstream in the shikimate pathway. The lack of a shift to EA/ETs with reduced nonACY flavonoid content may be due to the relative separation in their respective paths.

Although the absence of red color implies absence of anthocyanins, Simirgiotis *et al*. (2010) and Cheel *et al*. (2005) reported the presence of anthocyanin derivatives in white *F. chiloensis.* Similarly, the white *F. x ananassa* and *F. vesca* in the present work also contained anthocyanins, albeit at very low levels. Apart from anthocyanins, flavan-3-ols, mostly as B-type proanthocyanidins, were the major group of phenolic compounds in the *Fragaria* genotypes in the present study. Proanthocyanidins have been shown to constitute the major group of phenolic compounds apart from anthocyanins in *F. x ananassa* (Aaby *et al*., 2007, 2012; Carbone *et al*., 2009; Buendia *et al*., 2010), with the dimers such as B-type proanthocyanidins accounting for perhaps half of the total content (Aaby *et al*., 2012). Modulation of the regulatory transcription factors MYB1 and MYB10 in strawberry resulted in a decrease in anthocyanins (Medina-Puche *et al*., 2014) and increase in proanthocyanidins. The former but not the latter shift was evident in the white genotypes in this study. In fact, the white genotypes contained less B-type proanthocyanidins than the red genotypes within species (Table 4.5). Total anthocyanins (Table 4.3) and nonACYs (Fig. 4.2A) were significantly reduced in the white mutants of both species, suggesting a general reduction in phenylpropanoids/flavonoid enzyme activities. In addition, given the diverse content of EA/ETs of the white *F. vesca* cultivars, there were three genotypes that showed a possible re-direction of metabolite flux to EA/ETs, Baron Solemacher White, Yellow Wonder, and White Soul (Figuire 4.2A, B). Thus, among the white *F. vesca,* the variability in

the content of individual compounds implies differences in metabolite flux, indicative of, but not conclusive for, differing gene expression patterns for the enzymes within the phenylpropanoids/flavonoid biosynthetic pathway. Confirmation of this will require molecular analyses.

# Chapter 5: Developmental Variation in Fruit Polyphenol Content and Related Gene Expression of a Red- versus a White-Fruited Fragaria vesca Genotype

## 5.1 Introduction

The accumulation of diverse polyphenols during strawberry (*Fragaria* spp.) fruit ripening, including flavonols, proanthocyanidins, and especially anthocyanins, is responsible for enhancing fruit nutritional value and providing some level of defense against insects and pathogens (Lattanzio *et al*., 2006). Anthocyanins are also key to creating the bright red color of the ripe fruit. The types and levels of these secondary metabolites vary among commercial strawberry cultivars (*F. x ananassa* Duch.) (Buendia *et al*., 2010; Aaby *et al*., 2012;), as well as in the wild species *F. chiloensis* (Munoz *et al.,* 2011; Simirgiotis *et al.,* 2009) and *F. vesca* (Del Bubba *et al*., 2012). The variation among cultivars is due to genotype, cultural practices, and the environment (Josuttis *et al*., 2013).

Many of the major polyphenols in *Fragaria* are synthesized by the phenylpropanoid/flavonoid biosynthetic pathway. The genes and enzymes comprising the phenylpropanoid/flavonoid biosynthetic pathway have been characterized in octoploid *F. x ananassa* (Lunkenbein *et al*., 2006; Almeida *et al*., 2007; Griesser *et al*., 2008; Schwab *et al*., 2011). Because the complexity of the octoploid genome makes it difficult to study, the diploid woodland strawberry, *Fragaria vesca* (2n = 2x = 14), has become popular as an alternative to *F. x ananassa* because it has a small genome (240 Mb) (Folta and Davis, 2006), a short generation time, and an available full genome sequence (Kim *et al*., 2003; Slovin *et al*., 2009; Shulaev *et al.*, 2011). Though the fruit size of *F. vesca* is much smaller than of *F. x ananassa*, it is also a rich source of diverse polyphenols (Del Bubba *et al*., 2012; Sun *et al*., 2014) and commonly produces red fruit. Notably, there are some white-fruited cultivars, and some characterized as yellow, all lacking visible red color when ripe (Slovin *et al*., 2009; Sun *et al*., 2014).

In a prior study, polyphenol profiles of five white and one red cultivar of *F. vesca* were analyzed, exhibiting significant cultivar differences in metabolite content (Chapter 4). Major differences included the amount, and in some cases the absence, of anthocyanin in white berries. Recent studies have also shown differences between a red and a white *F.*

*vesca* in anthocyanin and ellagic acid/ellagitannin, non-flavonoid polyphenols, content (Gasperotti *et al*., 2013; Xu *et al*., 2014b). The major differences between red and white fruit were higher concentrations of conjugates of ellagic acids and ellagitannins in red *F. vesca,* and absence of anthocyanin in white berries of *F*. *vesca*. The complex process of strawberry fruit development and ripening is characterized by changes in size, color, and texture, with massive changes in accumulation of primary as well as secondary metabolites, and coordinated variation in transcript levels related to these events (Aharoni and O'Connell, 2002).

Although most of the basic biosynthetic steps leading to phenylpropanoid/flavonoid biosynthesis in *F. x ananassa* are known (Aharoni *et al*., 2001; Carbone *et al*., 2009), the regulation of these steps is not yet clearly established. Regulation of the components of the pathway are key to understanding metabolite flux and accumulation patterns. Several transcription factors (TFs) controlling the expression of the known flavonoid biosynthetic genes have been isolated and characterized in a variety of plant species. The known regulators of structural phenylpropanoid/flavonoid pathway genes are members of protein families containing R2R3 MYB domains, which are common in control of biosynthesis of all classes of flavonoids, and co-factors encoding the basic helix–loop–helix (bHLH) domains (also referred to as MYC proteins) and conserved WD repeats (WDR) have also been found (Stracke *et al*., 2001; Marles *et al*., 2003; Hichri *et al*., 2010, 2011).

The key TFs of phenylpropanoid/flavonoid pathway genes in many species including apple (*Malus X domestica* Borkh.) (Takos *et al*., 2006; Ban *et al*., 2007; Chagne *et al*., 2007; Lin-Wang *et al*., 2010), grape (*Vitis* spp.) (Kobayashi *et al*., 2004; Walker *et al*., 2007), peach (*Prunus persica*) (Rahim *et al*., 2014), nectarine (*Prunus persica*) (Ravaglia *et al*., 2013), and strawberry (Lin-Wang *et al*., 2010, 2014; Medina-Puche *et al*., 2014) leading to anthocyanin production are MYB10 and MYB1. Two MYB-genes, VvMYBA1 and VvMYBA2, were not functional in white grape berries (Boss *et al*., 1996), i.e., lacking anthocyanin, and a mutation in the promoter region of VvMYBA2 resulted in loss of anthocyanin biosynthesis in the skin and white berries (Kobayashi *et al*., 2004; Walker *et al*., 2007). MdMYBA/MdMYB1 and MdMYB10 controlled red pigmentation of apple. MdMYB1 and MdMYBA regulated anthocyanin accumulation in the skin, and MdMYB10 regulated the late pathway genes in both skin and flesh (Takos *et al*., 2006; Ban *et al*., 2007; Chagne *et al*., 2007; Lin-Wang *et al*., 2010). The phytohormone abscisic acid (ABA) may also play a

regulatory role in fruit ripening and anthocyanin biosynthesis of non-climacteric fruit like strawberry and grape (Peppi *et al*., 2008; Jia *et al*., 2011). ABA accumulation and expression of its key biosynthetic gene, 9-*cis*-epoxycarotenoid dioxygenase (VcNCED1), was correlated with anthocyanin accumulation in blueberry (Zifkin *et al*., 2012). Anthocyanin production was also inhibited with RNAi-mediated silencing of an ABA biosynthetic gene, FaNCED1, and a putative ABA receptor gene, magnesium chelatase H subunit (FaABAR/CHLH), in strawberry (Jia *et al*., 2011). In addition, Li *et al*. (2015) reported higher expression of anthocyanin-related genes in ABA-treated *F. x ananassa* berries.

In *F. chiloensis*, a comparison of anthocyanin profiles of a red cultivar and a white-fruited mutant indicated the absence of anthocyanins in the white mutant which was correlated with low expression of the anthocyanin-related biosynthetic genes (Salvatierra *et al*., 2010). Because the diploid *Fragaria vesca* genome is simpler than that of the octoploid *F. x ananassa* and *F. chiloensis*, *F. vesca* provides a more easily-defined model system with which to understand the molecular basis of the white mutation. The hypothesis for this study was that different metabolite profiles and transcriptional levels of one or more phenylpropanoid/flavonoid biosynthetic and regulatory genes would occur in white *F. vesca* when compared with red *F. vesca* during fruit development and ripening. Thus, analyses of the expression patterns of key structural genes of the phenylpropanoid/flavonoid biosynthetic pathway have been combined with high performance liquid chromatography-mass spectrometry (HPLC-MS)-based analyses of polyphenol profiles of a red and a white cultivar of *F. vesca* during fruit development and ripening to determine the basis of their color difference. For the polyphenol analyses at different stages of fruit development, a number of compounds were targeted to use as indicators of trends within specific groups of polyphenols. To explore whether transcription factors and ABA were also involved in anthocyanin synthesis, the expression patterns of MYB TFs, ABA biosynthetic genes, and a putative receptor gene throughout the fruit development were also studied.

## 5.2 Material and Methods

### 5.2.1 Chemicals and solvents

Gallic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, caffeic acid, ellagic acid, (+)-catechin, (+)-epicatechin, dihydroquercetin, quercetin, kaempferol, quercetin-3-glucoside and kaempferol-3-glucoside were purchased from Sigma Chemicals Co. (St. Louis,

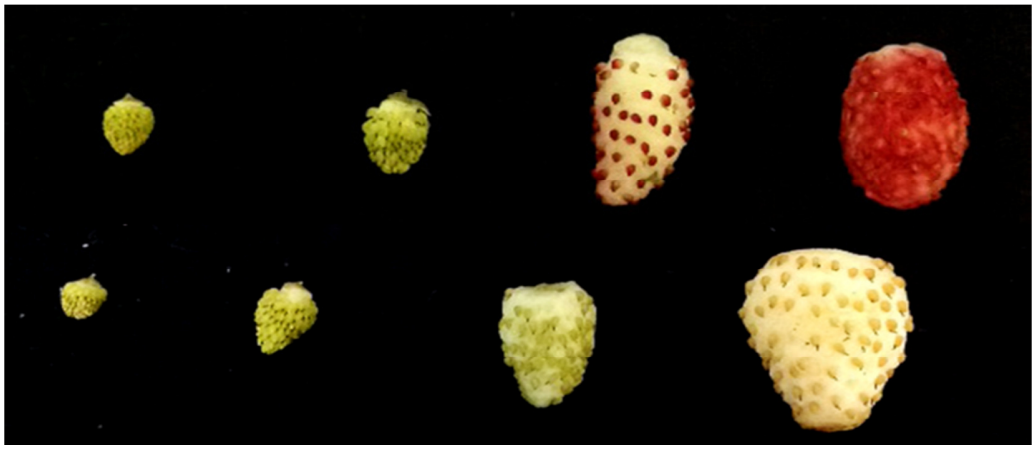
MO, USA). Pelargonidin chloride, pelargonidin-3-glucoside, and cyanidin-3-glucoside were obtained from Extrasynthase S.A. (Genay, France). Acetone, acetonitrile, methanol and chloroform were purchased from Fisher Scientific Co. (Tustin, CA). Water was of Milli-Q quality.

### 5.2.2 Plant material

Two cultivars of *F. vesca* were used in the study, red-fruited Baron Solemacher (BS) and white-fruited Pineapple Crush (PC) (Figure 5.1), chosen because they were consistent and abundant fruit producers. Eight or more plants of each cultivar were grown in 1.5-L containers in MetroMix 360 (Scotts, Marysville, OH, USA), and were watered and fertilized as needed. The plants were grown outdoors from March to November and in greenhouses from November to March in Lexington, KY. Fruit were harvested at four consecutive developmental stages: early green with no spacing between achenes (G1), intermediate green with spacing between achenes and a green receptacle exposed (G2), turning with the receptacle becoming pinkish-white for BS and white for PC (T), and ripe with a soft red receptacle for BS and soft white receptacle for PC (R). Berries were harvested as they were available, and were combined within harvest intervals of variable length and across plants within a genotype until sufficient biomass had been collected. Upon harvest, fruit were immediately frozen in liquid $N_2$ and stored at -80$^{o}$C until further use.

### 5.2.3 Extraction of phenolic compounds

Phenolic compounds were extracted from frozen fruit samples (3 replicates of 1 g from each developmental stage) which were first ground to a powder in a mortar and pestle using liquid nitrogen. The powders were extracted with 10 mL of extraction solution (acetone/water/acetic acid; 70:29.5:0.5, v/v/v) three times at 4 °C in the dark for 1 h per extraction (Simirgiotis *et al*., 2010), and the extraction volumes were combined. Combined extracts were centrifuged for 10 min at 3000 X g, and the supernatants were concentrated in a vacuum evaporator at 37°C (Kajdžanoska *et al*., 2011). The aqueous residues were filtered through an activated SepPak C-18 solid phase column and the phenolic content was eluted with acidified methanol (pH 3.4). The methanol solutions were evaporated at 37° C under reduced pressure (Kajdžanoska *et al*., 2010), residues were re-dissolved in 2 mL of 100% methanol, and were then filtered through a 0.45 μm nylon filter. Samples were stored at -80 °C until analysis.



Stage: G1 G2 T R

**Figure 5.1** Four developmental stages of baron solemacher (top row) and Pineapple Crush (bottom row). From left to right: early green (G1) with no spacing between achenes, intermediate green (G2) with spacing between achenes and green receptacle exposed, turning (T) with the receptacle becoming pinkish-white and achenes becoming red for BS and the receptacle becoming white for PC, and ripe (R) with a soft red receptacle for BS and soft white receptacle for PC.

### 5.2.4 LC/ESI-MS/MS analysis

The HPLC system consisted of dual Varian ProStar 210 pumps with a Varian ProStar 410 auto injector. Chromatographic separations were carried out on a 100 mm × 2.1 mm, 2.6 µm Phenomenex Kinetex C18 column. The mobile phase consisted of two solvents, water/formic acid (99.9%/0.1%, v/v) (solvent A) and methanol/formic acid (99.9%/0.1%, v/v) (solvent B). The gradient program entailed employing a linear gradient starting from 5% B at 0 min, to 50% B at 17 min, to 95% B at 22 min, then a return to 5% B at 24 min and constant 5% B for 10 min. The flow rate was 200 µL/min. An internal standard biochanin A was added to each sample prior to a run to ensure consistency of the retention times.

The mass detector was a Varian 1200L triple quadruple mass spectrometer with an electrospray ionization (ESI) interface controlled by Varian MS Data review (ver. 6.42) software. A 10 µL sample was injected into the HPLC-MS mobile phase and then directly into the ESI interface without splitting. All replicate samples were run in triplicate, in positive mode, followed by another positive mode, and then a negative mode interrupted by blank (solvent) injection to ensure no carryover between two runs. Nitrogen was used as the drying gas, and the capillary was maintained at 300 °C and voltage of 32V.

Quantification of the compounds was carried out using external standards. A calibration curve was made with pure compound, or a closely similar or basic unit of the compound when a pure reference standard was not available, at a range of concentrations, analyzed under the same conditions, and yielded linear regression coefficients greater than 0.9990. The specific external standards used are indicated in each table.

Final values of the content of each compound were corrected for loss during extraction by calculating the total anthocyanin as the sum of each individual anthocyanin detected and the mean total anthocyanin from 3 replicate extractions of fruit of each genotype in 80% MeOH (Chapter 3). This ratio of total anthocyanins from each method was used a correction factor across all compounds.

### 5.2.5 RNA isolation

Using berries collected during 3 separate harvest periods as biological replicates, total RNA was isolated according to the procedure of Reid *et al*. (2006) using a CTAB spermidine extraction buffer. The extraction buffer contained 300 mM Tris HCl (pH 8.0), 25

mM EDTA, 2 M NaCl, 2% CTAB, 0.05% spermidine trihydrochloride, and, just prior to use, 2% β-mercaptoethanol. The frozen tissue (5 g) was ground to a fine powder in a liquid nitrogen-filled mortar. The powder was added to pre-warmed (65°C) lysis buffer at 4 mL/g of tissue, and the mixture was transferred to a tube containing 2% PVPP (w/v) and shaken vigorously. Tubes were incubated in a 65°C water bath for 10 min and shaken every 2 min. Mixtures were extracted twice with equal volumes chloroform:isoamyl alcohol (24:1), then centrifuged at 3,500 X g for 15 min at 4°C. The aqueous layer was decanted to a new tube and centrifuged at 30,000 × g for 20 min at 4°C to remove any remaining insoluble material. Then, 0.1 mL 3 M NaOAc (pH 5.2) and 0.6 mL isopropanol were added to the supernatant, mixed, and incubated at -80°C for at least 30 min. The mixture was centrifuged at 3,500 X g for 30 min at 4°C to collect the pellets. The pellets were re-suspended in 1 mL TE (pH 7.5) and transferred to a microcentrifuge tube. To selectively precipitate the RNA, 0.3 mL of 8 M LiCl was added, and the sample was stored overnight at 4°C. The RNA was pelleted by centrifugation at 20,000 X g for 30 min at 4°C, washed with ice cold 70% ethanol, centrifuged in an eppendorf tube briefly, and decanted. Any residual ethanol was removed by air drying the tube, and then dissolved in 50–150 µL DEPC-treated water.

### 5.2.6 RNA purification, cDNA synthesis, and cloning of partial sequence of candidate genes

RNA quality was checked by assessing the 260/280 nm ratio before and after DNAse treatment, and RNA quantity was measured in a ND-1000 UV spectrophotometer (Nanodrop Technologies). Integrity of isolated RNA was checked on agarose gels stained with ethidium bromide. A DNAase treatment was made to remove contaminated genomic DNA, so 0.1 mL 10X TURBO DNAse buffer and TURBO DNAse was added to the extracted RNA. The mixture was incubated 30 min at 37°C, with 0.1 mL of DNAse inactivation reagent then added and incubated at room temperature for 5 min with mixing every 2 min. Finally, the mixture was centrifuged at the top speed of the eppendorf centrifuge for 5 min at 4°C, and the supernatant was collected.

First-strand cDNAs from each developmental stage of the fruit were synthesized from total RNA using the Thermo-Script RT-PCR System kit (Invitrogen) following manufacturer's instructions. The cDNAs were PCR amplified using gene specific primers (Table 5.1) for all developmental stages. The purified PCR products (ranging from 100 to

**Table 5.1** Primer sequence of the phenylpropanoid and flavonoid genes and housekeeping gene (actin) used for qRT-PCR. The primers were designed based on the gene transcripts with Gene IDs from a public database

| Target genes | Primers (F/R) | Amplicon size (bp) | Accession No: |
|---|---|---|---|
| PAL1 | F5'-CACAACATTACTCCCTGCTTGC-3'<br>R5'-CCTTTTGGTCCAACCGACTTAG-3' | 125 | XM_004304392.2 |
| C4H | F5'-GACGGTTCCTTTCTTCACCAAC-3'<br>R5'-AAGTTTCACGAACAGAGGGTCC-3' | 220 | DQ898278.1 |
| 4CL | F5'-AGAGCTCAAAGTCATCGAACCC-3'<br>R 5'-GAGCTCCTTAACCCTGTCAACG-3' | 217 | AF239685.1 |
| CHS | F5'- TGTGTGAGTACATGGCACCTTC-3'<br>R 5'-CCCATTCCTTAATGGCCTTG-3' | 105 | AY017477.1 |
| CHI | F 5'- ACAATGATACTACCGCTGACGG-3'<br>R 5'- CAATGGCTTTCGCTTCTGC-3' | 112 | AB201755.1 |
| F3'H | F5'-GAAGGACCTTTCGTGGTGAATC-3'<br>R5'-TGAGTTCTACATCCACGCACCT-3' | 247 | AY017479.1 |
| DFR | F-5'-CGGAGGGTGGTGTTTACATCTT-3' | 122 | AM691790.1 |

| | | | |
|---|---|---|---|
| | R-5’-CCAGTCATCTTTACTTTCCGGC-3’ | | |
| ANS | F5’-GCCTCAAACACCTTCCGACTAT-3’<br>R5’-TAACCCCCTCAGTTCCTTAGCA-3’ | 176 | AY017481.1 |
| LAR | F 5’-TTGAGAAGAGTGGGGTCCCTTA-3’<br>R 5’-GATCTGGAACTGATCCAACGGT-3’ | 116 | DQ087253.1 |
| UFGT1 | F 5’- CTGCTTATCGTGGCTTGACA-3’<br>R 5’- CCCGAAGTGACCACAAGAAT-3’ | 146 | AY695816.1 |
| MYB1 | F 5’- TTGCGTCGTTGTGGTAAGAG-3’<br>R5’- TCTGTCCTTCCAGGCAGTCT-3’ | 167 | AF401220.1 |
| MYB10 | F5’- TTGCAGGCTTAAACAGATGC-3’<br>F5’- CGCATGCTTTACCTGAGAGA-3’ | 207 | EU155163.1 |
| NCED1 | F5’- CTACTTCAACGGCAGGCTTCTT-3’<br>R5’- GTCGTATCTCCCTTCGGTTTTG-3’ | 100 | HQ290318.1 |
| CHLH/ABAR | F5’- GCGATCACAGTGTTCAGTTTCC-3’<br>R5’- CAAAGCGTCTGAAGTCTCTGGA-3’ | 168 | GQ201451.1 |
| Actin | F 5’- ACGAGCTGTTTTCCCTAGCA-3’<br>R 5’- CTCTTTTGGATTGAGCCTCG-3’ | 107 | AB116565.1 |

200 bp) were ligated into the pGEM-T-Easy Vector (Promega, USA), cloned and sequenced (Pattanaik *et al*., 2010). The resulting sequences were analyzed by BLASTp to access their homologies.

### 5.2.7 Quantitative real time RT–PCR

Expression patterns of each gene of interest were analyzed by RT-PCR, whereas transcript levels were determined by quantitative PCR (Pattanaik *et al*., 2010). For real time qPCR, 2 µg total RNA for each developmental stage was reverse-transcribed into single stranded cDNA using a SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, USA). cDNA was diluted 1:4, and 5 µL of the dilution was used for qPCR with gene specific primers and with 10 µL of iTaq SYBR Green Supermix with ROX (Bio-Rad, USA), on an Applied Biosystems StepOne Real-Time PCR System following thermal cycling conditions recommended by the manufacturer. Biological replicates were analyzed in triplicate. Specificity of amplification products was confirmed by the registration of a single peak in melting curves of the PCR products and the visualization of a single band on agarose gels. The actin gene with constant expression levels through all fruit developmental stages was used to normalize raw data and to calculate relative expression levels. G1 from Pineapple Crush was used as the calibrator sample in this study. A standard curve of 10-fold dilutions was considered for quantification of PCR amplification product. Confirmation of positive and specific amplification was performed with Table 1 dissociation curves. The specificity of each primer pair was verified by determining the melting curve of PCR products at the end of each run and by gel electrophoresis of the PCR products. The comparative Ct method (Applied Biosystems bulletin) was used to quantify the expression level of transcripts, setting all expressson levels against the value of Pineapple Crush at G1 within each gene. q-RT-PCR experiments were performed in triplicate (three technical replicates for each of the biological ones, in each developmental stage).

### 5.2.8 Statistical analyses

Polyphenol content was statistically analyzed for the main effects of genotype, stage of development, and their interaction by two-way ANOVA (SigmaPlot 12.0, Systat Software, Inc., San Jose, CA). Mean separation across gentoypes and stages was by Fisher's LSD at $P<0.05$. *t*-Tests at $P<0.05$ were used to compare relative expression levels of each gene between Baron Solemacher and Pineapple Crush at each stage of development.

## 5.3 Results and Discussion

### 5.3.1 Metabolic profiling of strawberry fruits at different development stages

#### 5.3.1.1 Flavonoid biosynthetic pathway metabolites

For the comparative analysis of flavonoid metabolites, the profiles of major flavonoids previously identified in ripe fruit (Chapter 4) were measured over four consecutive developmental stages in a red and a white *F. vesca*. There were significant effects ($P$ <0.001) of cultivar, stage of development, and their interaction on concentration of individual and total hydroxycinnamic acids (Table 5.2). Ferulic acid content of white Pineapple Crush fruit was more abundant at G1, G2, and R than of red Baron Solemacher, though they exhibited similar patterns with increases from G1 to G2 and T to R, and decreases from G2 to T. Both cholorogenic acid and *p*-coumaroyl hexose content were low in all stages of development, and no patterns were evident for either cultivar. Fait *et al*. (2008) reported ferulic acid and chlorogenic acid in the G2 stage were more abundant than *p*-coumaroyl hexose, which was greater in the ripe stages for red cultivars of *F. x ananassa*. Others have also reported that derivatives of *p*-coumaroyl acids increased from early to ripe stages in *F. x ananassa* (Carbone *et al*., 2009; Aaby *et al.*, 2012). Total hydroxycinnamic acid content was comprised primarily of ferulic acid and reflected the differences described for it.

In our previous work with one red and five white cultivars of *F. vesca*, free forms of caffeic acid and *p*-coumaric acid were detected but ferulic acid was not found, in contrast to the present results. Hydroxylated and/or methylated conjugates of hydroxycinnamic acid can be readily synthesized from *p*-coumaric acid in the presence of *p*-coumaroyl shikimate/quinate-3'-hydroxylase (C3'H) to form caffeic acid, and caffeic acid can further be methylated by caffeic acid/5-hydroxyferulic acid–*O*-methyltransferase (COMT) to form ferulic acid (Nair *et al*., 2004). Because the polyphenol composition of strawberry fruit has been shown to depend also on factors such as genotype, maturity stage, production site, environmental effects, and even extraction solvent and analytical method (Kosar *et al*., 2004; Bacchella *et al*., 2009, Carbone *et al*., 2009; Kajdžanoska *et al*., 2011;

**Table 5.2** Changes in the content of individual and total hydroxycinnamic acids during development and ripening of the *Fragaria vesca* cultivars Baron solemacher and Pineapple Crush with red and white fruit, respectively.

| Cultivar | Developmental stage[z] | FA[y] | ChA | PCH[x] | Total |
|---|---|---|---|---|---|
| | | (mg · 100 $g^{-1}$ fresh weight) | | | |
| Baron | G1 | 3.9 ± 0.2 f[w] | 0.3 ± 0 e | 0.4 ± 0 a | 4.6 ± 0.2f |
| Solemacher | G2 | 14 ± 0.8 c | 0.7 ± 0 a | 0.2 ± 0 c | 14.9 ± 0.8 c |
| | T | 4.9 ± 0.1 ef | 0.6 ± 0 b | 0.2± 0 bc | 5.7 ± 0 ef |
| | R | 10.9 ± 0.04 d | 0.09±0 g | 0.3 ± 0 b | 11.2 ± 0 d |
| Pineapple | G1 | 26.1 ± 0.3 b | 0.4 ± 0 d | 0.2 ± 0c | 26.7± 0.3 b |
| Crush | G2 | 38.7 ± 1.8 a | 0.2 ± 0 f | 0.2 ± 0 c | 39.1± 1.8 a |
| | T | 7.3 ± 0.3e | 0.5 ± 0 c | 0.2± 0 c | 8 ± 0.2 e |
| | R | 14.7 ± 0.9 c | 0.4 ± 0 d | 0.3 ± 0 b | 15.4 ± 0.9 c |
| | | | ANOVA (*P*) | | |
| Cultivar | | <0.001 | NS | <0.001 | <0.001 |
| Stage | | <0.001 | <0.001 | <0.001 | <0.001 |
| Cultivar X Stage | | <0.001 | <0.001 | <0.001 | <0.001 |

[z]Developmental stages are: G1=early green with no spacing between achenes, G2=intermediate green with spacing between achenes and green receptacle exposed,

Table 5.2 (continued)

T=turning with the receptacle becoming pinkish-white for BS and white for PC, and R=ripe with a soft red receptacle for BS and soft white receptacle for PC.

[y]Compound abbreviations: FA: ferulic acid; ChA: cholorogenic acid; PCH: *p*-coumaric acid hexose.

[x]PCH quantified as *p*-coumaric acid.

[w]Means (*n=3*) ± SD in the same column followed by different letters are significantly different by Fisher's LSD at $P<0.05$. SD = 0 if SD≤0.05 mg/100 g fresh weight. NS=not significant.

Doumett *et al*., 2011; Josuttis *et al*., 2013), the differing results may be due to one or more of these factors, most likely production environment.

The flavonols in *Fragaria* spp. are comprised mainly of derivatives of quercetin and kaempferol. Kaempferol-3-glucoside was highest in Baron Solemacher at G1 and declined during development, while it increased from G1 to T before declining at R in Pineapple Crush (Table 5.3). Kaempferol acetyl glucoside varied with no clear patterns in both cultivars from G1 to T but increased in both at R. Kaempferol coumaroyl hexose was highest at G2 and lowest at R of both cultivars. Kaempferol acetylglucose was only found in the ripe stage of red cultivars of *F. x ananassa* with greater accumulation of the glucoside and coumaroyl glucose derivatives of kaempferol in the later than the earlier stages (Fait *et al*., 2008). Quercetin was not detected at some stages, and more quercetin-3-glucoside was evident at all stages in both red and white cultivars. Quercetin-3-glucoside was generally greater at the earliest stage and subsequently declined in both cultivars as well. Total flavonol content was greater at G1 and declined by stage T in Baron Solemacher, but it was constant from G1 to T before declining at R in Pineapple Crush.

Among flavan-3-ols, catechin and proanthocyanidin dimer content was 6- to 200-fold higher than epicatechin content across cultivars and stages, respectively (Table 5.4). The content of flavan-3-ols in both genotypes increased from G1 to G2, then declined until ripening. Total flavan-3-ol content of Pineapple Crush was significantly lower than for Baron Solemacher at R, as observed in an earlier study (Chapter 3). Published data on flavan-3-ol content of *F. vesca* is not available, but several studies with red-fruited *F. x ananassa* showed a decreasing level of epicatechin, catechin and proanthocyanidin content throughout development (Almeida *et al*., 2007; Carbone *et al*., 2009).

Anthocyanin derivatives were by far the primary flavonoids to show the most contrasting results between red and white *F. vesca* fruit, as previously shown (Chapter 4). Six derivatives of pelargonidin, cyanidin and peonidin were found at the T and R developmental stages of Baron Solemacher, but none were detected in Pineapple Crush (data not shown). Pelargonidin-3-glucoside was the major anthocyanin, followed by cyanidin-3-glucoside. Very low levels of pelargonidin-malonyl glucoside, cyanidin-malonyl glucoside, peonidin-3-glucoside, and peonidin-malonyl glucoside were also detected. Total anthocyanin content for Baron Solemacher was 55 mg · 100 $g^{-1}$ fresh weight in the present

**Table 5.3** Changes in content of individual and total flavonols during development and ripening of the *Fragaria vesca* cultivars Baron solemacher and Pineapple Crush with red and white fruit, respectively.

| Cultivar | Developmental stage[z] | K3G[y] | KAG | KCH[x] | Q | Q3G | Total |
|---|---|---|---|---|---|---|---|
| | | (mg · 100 $g^{-1}$ fresh weight) | | | | | |
| **Baron** | G1 | 5.5 ± 0.1[w] b | 1.4 ± 0 de | 3.1 ± 0 ab | Nd | 12.1 ± 0.2 a | 22.1 ± 0.2 a |
| **Solemacher** | G2 | 4.2 ± 0 c | 1.4± 0 de | 3.3 ± 0 a | 0.3 ± 0 ab | 8.5 ± 0.2 b | 17.7 ± 0.2 b |
| | T | 1.8 ± 0 e | 1.2 ± 0 e | 1.8 ± 0 d | Nd | 4.1 ± 0 e | 8.9± 0 e |
| | R | 1.1 ± 0 f | 1.9 ± 0 bc | 1.1 ± 0 e | 0.2 ± 0b | 4.4 ± 0 e | 8.7 ± 0.1 e |
| **Pineapple** | G1 | 3.8 ±0 cd | 1.4 ± 0 de | 2.4 ± 0 c | 0.3 ± 0ab | 6.1 ± 0 d | 14 ± 0.2 c |
| **Crush** | G2 | 4.6± 0b | 2.2 ± 0 b | 3 ± 0 b | 0.7 ± 0 a | 3.4 ± 0 f | 13.9 ± 0.2 c |
| | T | 6.2 ± 0 a | 1.5 ± 0 cd | 2.4 ± 0 c | Nd | 3.6 ± 0 f | 13.7 ± 0.2 c |

**Table 5.3 (continued)**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | R | 1.6 ± 0 ef | 2.8 ± 0 a | 1.1± 0 e | 0.2 ± 0 a | 4.3± 0 e | 10 ± 0.2 d |
| | | | | ANOVA (*P*) | | | |
| **Cultivar** | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Stage** | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Cultivar X Stage** | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

[z]Developmental stages are: G1=early green with no spacing between achenes, G2=intermediate green with spacing between achenes and green receptacle exposed, T=turning with the receptacle becoming pinkish-white for BS and white for PC, and R=ripe with a soft red receptacle for BS and soft white receptacle for PC.

[y]Compound abbreviations: K3G: kaempferol-3-glucoside; KAG: kaempferol acetyl glucose; KCH: kaempferol coumaroyl hexose; Q: quercetin; Q3G: quercetin-3-glucoside.

[x]KCH quantified as kaempferol-3-glucoside.

[w]Means (*n=3*) ± SD in the same column followed by different letters are significantly different by Fisher's LSD at P<0.05. SD = 0 if SD≤0.05 mg/100 g fresh weight. nd indicates it was not detected.

**Table 5.4** Changes in content of the catechin, epicatechin and proanthocyanidin during development and ripening of the *Fragaria vesca* cultivars Baron solemacher and Pineapple Crush with red and white fruit, respectively.

| Cultivars | Developmental stages[z] | Catechin[y] | Epicatechin | PCD[x] | Total |
|---|---|---|---|---|---|
| | | mg · 100 $g^{-1}$ fresh weight | | | |
| Baron | G1 | 50 ± 1 e[w] | 1.6 ± 0 f | 210± 1 f | 260 ± 1 f |
| Solemacher | G2 | 115± 1 a | 4.6 ± 0 b | 484± 8 c | 604 ± 8 b |
| | T | 93 ± 2 b | 3.6 ± 0 d | 460± 8 d | 558 ± 9 c |
| | R | 86 ± 6 c | 4 ± 0 c | 326± 1 e | 417± 5 d |
| Pineapple | G1 | 61 ± 1 d | 2.5 ± 0 e | 213 ± 4 f | 277 ± 3 e |
| Crush | G2 | 114 ± 1 a | 5.8± 0 a | 545± 5 a | 665 ± 9a |
| | T | 96 ± 0 b | 3.6 ± 0 d | 505 ± 6b | 605 ± 6 b |
| | R | 40 ± 0 f | 2.6± 0 e | 167 ± 1 g | 210 ± 1 g |
| | | | ANOVA (*P*) | | |
| Cultivar | | <0.001 | <0.001 | <0.001 | <0.001 |
| Stage | | <0.001 | <0.001 | <0.001 | <0.001 |
| Cultivar X Stage | | <0.001 | <0.001 | <0.001 | <0.001 |

[z]Developmental stages are: G1=early green with no spacing between achenes, G2=intermediate green with spacing between achenes and green receptacle exposed,

Table 5.4 (continued)

T=turning with the receptacle becoming pinkish-white for BS and white for PC, and R=ripe with a soft red receptacle for BS and soft white receptacle for PC.

[y]Compound abbreviations: CAT: catechin; EPI: epicatechin; PCD: proanthocyanidin dimers; TPA: total proanthocyanidin.

[x]Quantified as catechin.

[w]Means (*n=3*) ± SD in the same column followed by different letters are significantly different by Fisher's LSD at $P<0.05$. SD=0 if SD≤0.05 mg/100 g fresh weight.

study. In other red *F. vesca* it has varied from 15 to over 120 mg · 100 $g^{-1}$ fresh weight (Cheel *et al*., 2007; Najda *et al.*, 2014; Yildiz *et al*., 2014). These differences among our studies and those of others may be further evidence of the significant impact of genotype, environment, and production practices on the accumulation of secondary metabolites in fruit of *Fragaria* spp. (Carbone *et al*., 2009; Bacchella *et al.*, 2009; Josuttis *et al*., 2013).

#### 5.3.1.2 Ellagic acid, its derivatives, and ellagitannins

Another major group of polyphenols in *Fragaria* spp. are ellagic acid, its derivatives, and ellagitannins, which are synthesized by a branch of the shikimate pathway from a gallic acid precursor (Muir *et al*., 2011). Free EA content was highest in the early stages of both cultivars and then decreased (Table 5.5), as reported by Gasperotti *et al*. (2013). Among derivatives of ellagic acid, EA deoxyhexoside showed higher accumulation than the other EA derivatives, methyl EA pentoside and EA pentoside. EA deoxyhexoside showed a decreasing level of product accumulation from G1 to T stages of both fruit color types. Gasperotti *et al*. (2013) did not detect EA deoxyhexoside in red or white fruit of *F. vesca*, but content was high in G1 and G2 of red fruit of *F. x ananassa*. Total EA derivative content was highest at the earliest stages of development of both cultivars.

Two ellagitannins, hexahydroxydipenoyl (HHDP) hexoside and galloyl HHDP hexose, were abundant in the fruit throughout the developmental process (HGH and GHH, respectively, in Table 5.6). Overall, both ellagitannins declined from stage G1 to R, but at R Pineapple Crush had more total ellagitannin. Fait *et al.* (2008) also reported a higher content of ellagitannins in achenes and receptacle of strawberry in the earlier stages of fruit development. Gasperotti *et al*. (2013) reported that the total content of 26 identified ellagic acid derivatives and ellagitannins was considerably higher than of the 2 studied in this work, and that red *F. vesca* had a greater content than white when ripe. However, trends over time were the same as reported here.

The accumulation pattern of total polyphenols reflects two different directions of carbon flow for the non-anthocyanin flavonoids versus the ellagic acid derivatives/ellagitannins in red and white fruited *F. vesca* cultivars. Although the content of total hydroxycinnamic acids (Table 5.2) and flavan-3-ols (Table 5.4) were greater in earlier

**Table 5.5** Changes in individual and total ellagic acid derivatives content during development and ripening of the *Fragaria vesca* cultivars Baron solemacher and Pineapple Crush with red and white fruit, respectively.

| Cultivars | Developmental stages[z] | EA[y] | EAD[x] | MEAP | EAP | Total |
|---|---|---|---|---|---|---|
| | | mg · 100 $g^{-1}$ fresh weight | | | | |
| **Baron Solemacher** | G1 | 86 ± 1 b[w] | 581 ± 4 a | 44 ± 1b | 107 ± 0 b | 2887± 9 a |
| | G2 | 104 ± 2 a | 575 ± 3a | 33 ± 0 cd | 117 ± 3 a | 3232 ± 33 a |
| | T | 30 ±2 f | 245 ± 5 e | 35 ± 0 c | 53 ± 3 g | 1524 ± 17 f |
| | R | 33 ± 1 f | 254 ± 5 e | 46 ± 0b | 77 ± 1 e | 1421 ± 4e |
| **Pineapple Crush** | G1 | 108 ± 1 a | 571 ± 5a | 25 ± 1e | 110 ± 2 b | 2910 ±25a |
| | G2 | 56± 2 c | 424 ± 2 b | 29± 0 de | 67 ± 1 f | 2384 ± 2b |
| | T | 37 ± 0 e | 359 ± 4 c | 44 ± 4b | 87 ± 10d | 2223 ± 3c |

**Table 5.5 (continued)**

| | | | | | | |
|---|---|---|---|---|---|---|
| | R | 44 ± 1 d | 308 ± 5 d | 53 ± 1 a | 93 ± 1 c | 2381 ± 7d |
| | | | | ANOVA (*P*) | | |
| **Cultivar** | | NS | NS | NS | NS | <0.001 |
| **Stage** | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Cultivar X Stage** | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

[z]Developmental stages are: G1=early green with no spacing between achenes, G2=intermediate green with spacing between achenes and green receptacle exposed, T=turning with the receptacle becoming pinkish-white for BS and white for PC, and R=ripe with a soft red receptacle for BS and soft white receptacle for PC.

[y]Compound abbreviations: EA, ellagic acid; EAD, EA deoxyhexoside; MEAP, methyl EA pentose; DEAP, deoxyEA pentose; EAP, EA pentose; TEAD, total ellagic acid derivatives.

[x]Quantified as ellagic acid. [w]Means (*n=3*) ± SD in the same column followed by different letters are significantly different by Fisher's LSD at P<0.05. SD=0 if SD≤0.05 mg/100 g fresh weight. NS=not significant.

**Table 5.6** Changes in individual and total ellagitannin content during development and ripening of *Fragaria vesca* cultivars Baron solemacher and pineapple Crush with red and white fruit, respectively.

| Cultivars | Developmental stages[z] | HGH[yx] | GHH | TET |
|---|---|---|---|---|
| | | mg · 100 $g^{-1}$ fresh weight | | |
| Baron Solemacher | G1 | 536 ± 5 a[zw] | 217± 3 a | 757 ± 7a |
| | G2 | 531 ± 8 a | 151 ± 5 d | 686 ± 8 b |
| | T | 123 ± 1 e | 83± 3 g | 212 ± 3 f |
| | R | 92 ± 1 f | 43 ± 1 h | 140 ± 1 g |
| Pineapple Crush | G1 | 477 ± 3 b | 161 ± 4 c | 643 ± 2 c |
| | G2 | 371 ± 5c | 178 ± 4 b | 554 ± 8 d |
| | T | 214 ± 2d | 125± 3 e | 344 ± 4 e |
| | R | 216 ± 4 d | 114 ± 2 f | 336 ± 6 e |
| | | | ANOVA (*P*) | |
| Cultivar | | <0.001 | <0.001 | <0.001 |
| Stage | | <0.001 | <0.001 | <0.001 |
| Cultivar X Stage | | <0.001 | <0.001 | <0.001 |

[z]Developmental stages are: G1=early green with no spacing between achenes, G2=intermediate green with spacing between achenes and green receptacle exposed,

Table 5.6 (continued)

T=turning with the receptacle becoming pinkish-white for BS and white for PC, and R=ripe with a soft red receptacle for BS and soft white receptacle for PC.

[y]Compound abbreviations: HGH, HHDP galloyl hexose; GHH, Galloyl HHDP hexose; AR, argimonin; TET, total ellagitannin

[x]Quantified as ellagic acid.

[w]Means (*n=3*) ± SD in the same column followed by different letters are significantly different by Fisher's LSD at $P<0.05$. SD=0 if SD≤0.05 mg/100 g fresh weight.

stages of development in white Pineapple Crush, values were lower at the final R stage than for red Baron Solemacher. However, the opposite was true for ellagic acid derivatives (Table 5.5) and ellagitannins (Table 5.6). A significant reduction in the total non-anthocyanin flavonoids at the ripe stage were also found in five white-fruited *F. vesca* cultivars in our previous study (Chapter 4).

### 5.3.2 Transcriptional profiles of the structural genes of the phenylpropanoid /flavonoid biosynthetic pathway in red versus white *Fragaria vesca* fruit during development

Transcriptional profiles were analyzed for the structural genes of the phenylpropanoid/flavonoid biosynthetic pathway at the four developmental stages, G1, G2, T, and R (Figure 5.1), of the red- and white-fruited *F. vesca* cultivars. The early biosynthetic pathway consists of three enzymes, phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL), which are responsible for providing the precursors leading to the production of polyphenolic compounds such as lignins, anthocyanins and other flavonoids. At the G1 and G2 stages, the first phenylpropanoid pathway gene PAL, the enzyme responsible for catalyzing the trans-elimination of ammonia from phenylalanine and producing *trans*-cinnamic acid (Salvatierra *et al*., 2010), showed similar expression in the red and white cultivars, but at the T stage PAL was higher in Baron Solemacher than Pineapple Crush, though transcript abundance had declined in both (Fig. 5.2). PAL was significantly upregulated in red Baron Solemacher at the final R stage, but there was no change in Pineapple Crush. Salvatierra *et al*. (2010) did not see a difference in PAL expression between red and white genotypes of *F. chiloensis*.

Few differences between cultivars were observed in the expression patterns of C4H, the enzyme responsible for catalyzing the 4-hydroxylation of *trans*-cinnamate, and 4CL. C4H was greater in Baron Solemacher at T only, and 4CL was greater in Pineapple Crush at G1 only. The next enzyme in the pathway, CHS, catalyzes the entry step into the flavonoid biosynthetic pathway from the phenylpropanoid pathway by converting 4-coumaroyl-CoA and three molecules of malonyl-CoA into naringenin chalcone. The CHS transcript level

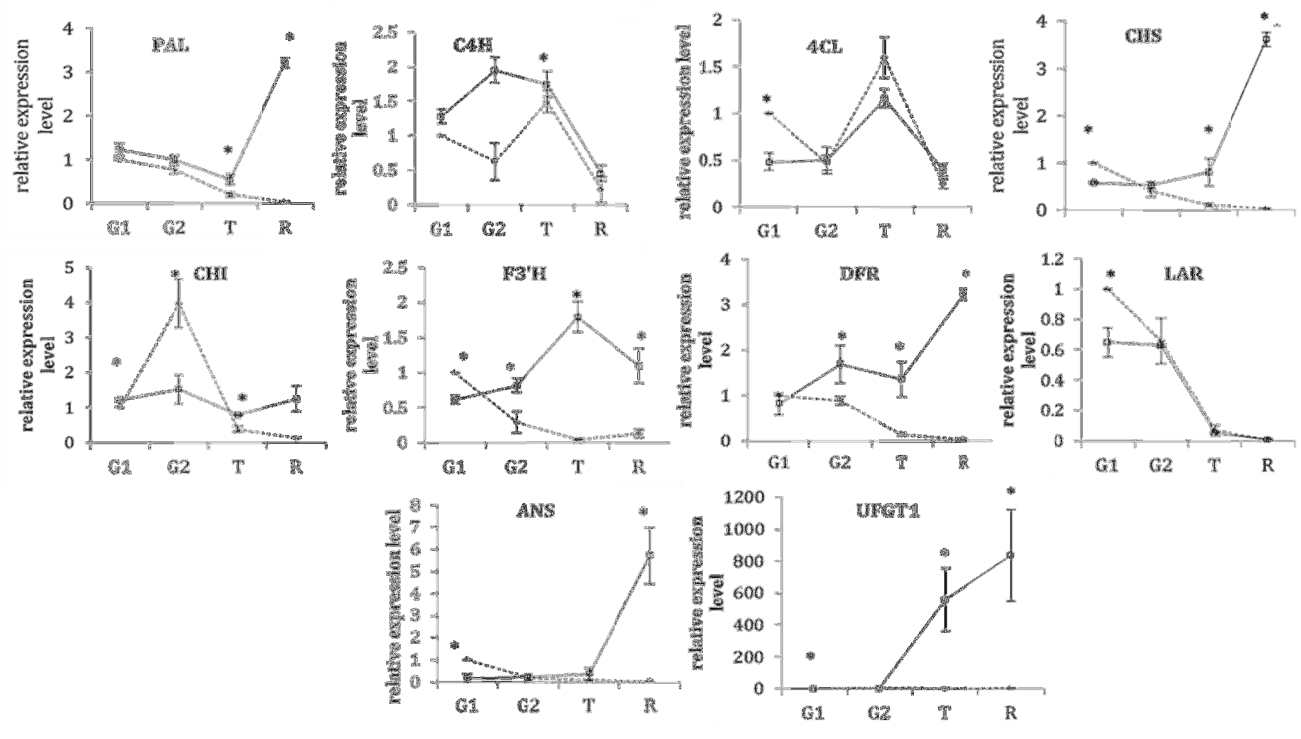



**Figure 5.2** Transcript levels of structural genes involved in the phenylpropanoid/flavonoid biosynthetic pathway during development and ripening of the Fragaria vesca cultivars, Baron Solemacher (solid line) and Pineapple Crush (dashed line) with red and white fruit, respectively. Developmental stages are: G1=early green with no spacing between achenes, G2=intermediate green with spacing between achenes and green receptacle exposed, T=turning with the receptacle becoming pinkish-white for BS and white for PC, and R=ripe with a soft red receptacle for BS and soft white receptacle for PC. Data represented are the mean of three individual experiments with error bars indicating ± SD. Asterisks indicate significant difference as determined by Student's t-test ($P<0.05$).

was greater at G1 in Pineapple Crush, did not differ between cultivars at G2, but was greater in Baron Solemacher at T and R. Similar patterns were observed during fruit development of red and white *F. vesca* fruit (Xu *et al*., 2014b), red and white forms of *F. chiloensis* (Salvatierra *et al*., 2010), and in several red *F. x ananassa* genotypes (Almeida *et al*., 2007; Carbone *et al*., 2009; Saud *et al*., 2009).

The transcript abundance of CHI was significantly higher in the G2 stage of Pineapple Crush, but less than Baron Solemacher at stages T and R, in agreement with Xu *et al*. (2014b) for red versus white *F. vesca*. The abundance of F3'H transcript was significantly different in all stages of the fruit development, with Pineapple Crush greater than Baron Solemacher at stage G1, but less at all subsequent stages. The expression profile of DFR in red *F. vesca* was greater than of the white form from G2 to R stages, with an increase from T to R in the red and a decline in the white. These patterns for transcript abundance of F3'H and DFR were also reported for red and white *F. vesca* by Thill *et al*. (2013) and Xu *et al*. (2014b).

After DFR, transcript abundance for the biosynthetic gene specific for proanthocyanidin production, LAR, decreased from G1 to R for both red and white cultivars. Interestingly, in the initial green stage G1, the expression level was significantly higher in the white Pineapple Crush than in the red Baron Solemacher, as reported by Xu *et al.* (2014b). In contrast to LAR transcript abundance, the anthocyanin-related genes ANS and UFGT1 were significantly upregulated late in fruit development in Baron Solemacher only, at R for ANS and at T and R for UFGT1. UFGTs catalyze transfer of glucose from UDP-activated sugar donor molecules to flavonols and anthocyanidins which increases water solubility and storability of the stabilized flavonoid compounds in the vacuoles. UFGT acts more as a modifying enzyme for anthocyanins (Salvatierra *et al*., 2010), and can be considered one of the key enzymes determining the accumulation of anthocyanin glycosides and the hue of fully ripe fruit. Silencing of FaGT1 in *F. x ananassa* showed reduced levels of pelargonidin 3-glucoside malonate and pelargonidin 3-glucoside (Griesser *et al.*, 2008). In the present study, there were very low but detectable levels of UFGT transcript throughout fruit development in white Pineapple Crush.

### 5.3.3 Transcriptional profiles of key transcription factors of the flavonoid biosynthetic pathway in red versus white *Fragaria vesca* fruit during development

The expression profiles of the transcription factors (TFs) MYB1 and MYB10 (Figure 5.3) were most notably significantly lower at the R stage in Pineapple Crush than in Baron Solemacher. Medina-Puche *et al*. (2014) suggested that MYB10 may regulate most or all of the flavonoid pathway genes in the early and late stages of fruit development in *F. x ananassa*. There was no apparent expression of MYB10 at G1 and slight expression at G2 for both red and white genotypes in our study, as with FaMYB10 in *F. x ananassa* (Medina-Puche *et al*., 2014). Lin-Wang *et al*. (2014) showed that overexpression of FvMYB10 in *F. vesca* greatly increased anthocyanin concentration, but other flavonoid levels were not affected except for *p*-coumaroyl glucose, suggesting MYB10 may only act on the final anthocyanin-related branch. However, FaMYB10 and FvMYB10 may have different roles in regulating ANS as FaMYB10 silenced lines had unchanged ANS levels, which indicated that regulation of ANS was not dependent on FaMYB10 (Medina-Puche *et al.*, 2014). In contrast, only heavily FvMYB10-silenced lines exhibited downregulation of ANS gene expression along with expression of CHS, F3'H, DFR and UFGT (Lin-Wang *et al*., 2014; Medina-Puche *et al*., 2014). MYB10 was lower in Pineapple Crush at stage R in comparison to Baron Solemacher with downregulation of anthocyanin biosynthesis genes ANS and UFGT1 suggesting that MYB10 may regulate these anthocyanin-related genes in *F. vesca*.

The MYB1 transcript levels in this study differ from those for FaMYB1 in red *F. x ananassa,* increasing at R rather than declining (Lin-Wang *et al*., 2010), and for FcMYB1 in a white *F. chiloensis* where it was high at T and R, suppressing anthocyanin accumulation (Salvatierra *et al*., 2013). In contrast to the present results, FvMYB1 showed little change throughout development (Lin-Wang *et al*., 2010).

Perhaps other TFs, MYB10 isoforms, or co-factors exist that need to be identified for a full understanding of regulation of flavonoid biosynthesis in *F. vesca*. In a recent study by Starkevič *et al.* (2015), the homologs PaMYB10.1 and PaMYB10.2 were isolated in sweet cherry (*Prunus avium*), where subvariant gene PaMYB10.1-1 of variant PaMYB10.1 showed higher expression in fruit with higher anthocyanin content and was highly correlated with the expression of PaUFGT, whereas subvariant PaMYB10.1-3 showed low levels of expression in fruit. Another expression analysis of 4 candidate MYB transcription factors,

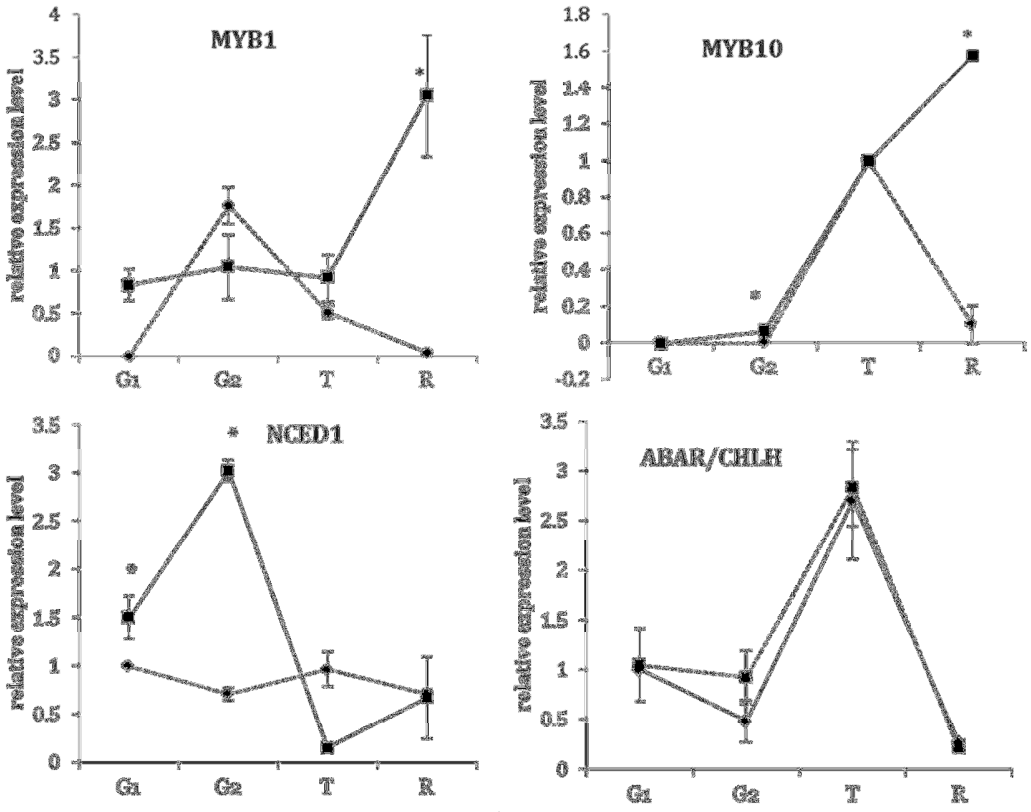



**Figure 5.3** Transcript abundance of the key transcription factors, MYB1 and MYB10, and of the abscisic acid biosynthesis and receptor genes, NCED1 and ABAR/CHLH, respectively, during development and ripening of the *Fragaria vesca* cultivars, Baron Solemacher (soild line) and Pineapple Crush (dashed line) with red and white fruit, respectively, by qRT-PCR. Developmental stages are: G1=early green with no spacing between achenes, G2=intermediate green with spacing between achenes and green receptacle showing, T=turning with the receptacle becoming pinkish-white for BS and white for PC, and R=ripe with a soft red receptacle for BS and soft white receptacle for PC. Data represented are the mean of three individual experiments with error bars indicating ± SD. Asterisks indicate a significant difference as determined by Students's t-test ($P$<0.05).

homologous to those in *P. avium* L., MYB10, MYB11, MYB111 of apple (*Malus* x *domestica*) and a putative MYB transcription factor of *Rosa rugosa* showed a higher transcript accumulation in red sweet cherry in comparison to the yellow fruit in the later fruit development stages (Wei *et al*., 2015). In *F. x ananassa*, two regulatory genes, FaMYB9 and FaMYB11, interacted with FaTTG1 to regulate proanthocyanidin accumulation during early stages of fruit development (Schaart *et al*., 2013). The expression of FaANS was influenced by FaMYB5 but not by FaMYB10, although this needs further confirmation. These observations suggest complex role of MYBs in the regulation of anthocyanin and other flavonoid biosynthesis. Further work is required to determine if any other homologs of MYB10 may play a direct regulatory role in anthocyanin production in *F. vesca*

### 5.3.4 Transcriptional profiles of ABA-related genes in strawberry fruit at different developmental stages

A significantly higher transcript abundance of the key ABA biosynthetic gene NCED1 was observed at stages G1 and G2 of Baron Solemacher, though it declined by T and R and was similar to Pineapple Crush (Fig. 5.3), suggesting a decline in the biosynthesis of ABA when fruit are starting to ripen. In contrast, in Pineapple Crush, the transcript levels of the ABA biosynthetic gene NCED1 varied little during fruit development. In *F. x ananassa*, FaNCED1 generally increased during development (Jia *et al*., 2011), correlated to the increase in fruit ABA content. There were no differences in the expression patterns for the ABA receptor gene ABAR/CHLH between the red and white cultivars, although the transcript abundance in both was upregulated at G2 prior to the ripening process commencing. In contrast, transcript levels of FaABAR/CHLH were higher in green fruit than in turning and red fruit in *F.* x *ananassa* (Jia *et al*., 2011). *F. x ananassa* fruit agroinfiltrated with FaNCED1-RNAi constructs or treated with the ABA inhibitor nordihydroguaiaretic acid (NDGA) showed lack of red color development and a decrease in FaMYB10 transcript levels (Medina-Puche *et al*., 2014). Expression of FAMYB10 was correlated with the presence of ABA in the fruit. In white Pineapple Crush, NCED1 transcript levels were not related to lack of color development as others have reported.

### 5.3.5 Conclusions

The accumulation and distribution of polyphenols are governed by a metabolic network that is strongly connected to and co-regulated with the expression of structural

and regulatory genes of the flavonoid biosynthetic pathway (Almeida *et al*., 2007; Carbone *et al*., 2009; Pillet *et al*., 2015). Combined metabolite profiling and transcriptional studies of the key genes of the multiple-routed pathway with a diversity of end products were performed to provide insight into the patterns of metabolite flux during fruit development in red- versus white-fruited *F. vesca*. Expression studies of octoploid strawberry (*F. x ananassa*) showed biphasic upregulation patterns for CHS, CHI, LAR and ANS during fruit development (Halbwirth *et al*., 2006; Almeida *et al*., 2007; Carbone *et al*., 2009), with high expression at the early stages of development followed by a subsequent decrease, and upregulation again at the T stage. In the present study, this pattern was not observed. Rather, the present results generally agree with those for red- and white-fruited *F. vesca* reported by Xu *et al*. (2014b), that red *F. vesca* expression patterns were variable among key genes but no dominant pattern was evident.

There is a growing body of evidence that downregulation of key enzymes of the phenylpropanoid/flavonoid pathway in *Fragaria* spp. (Cheel *et al.*, 2005, 2007; Fischer *et al*., 2014; Greisser *et al*., 2008; Jiang *et al.*, 2012; Lin-Wang *et al.*, 2014; Lunkenbein *et al.*, 2006; Ring *et al.*, 2013; Saud *et al.*, 2009) results in re-direction of metabolites within the pathways. In the present work, transcript abundance of both PAL and CHS in the phenylpropanoid pathway, were significantly lower in white Pineapple Crush than red Baron Solemacher fruit at stages T and R as anthocyanin production was increasing in the latter genotype (Fig. 1). Although the two enzymes in the biosynthetic pathway between PAL and CHS, C4H and 4CL, did not show clear patterns or differences between the red and white forms, greater levels of ferulic acid, chlorogenic acid, and *p*-coumaric acid in Pineapple Crush (Table 2) may indicate a re-direction of the pathway to hydroxycinnamic acids from the flavonoid pathway due to reduced expression of PAL and CHS. Similarly, in white-fruited *F. chiloensis*, lack of C4H expression was accompanied by increased hydroxycinnamic compounds (Cheel *et al.*, 2005, 2007; Saud *et al.*, 2009).

Both F3'H and DFR in the flavonoid pathway were significantly lower from stages G2 to R in the white than the red fruit genotype (Fig. 5.2). However, the total flavonol pool was greater in the white than the red genotype (Table 5.3), suggesting the differences in transcript abundance were not affecting metabolite flux in the pathway at this point. Proanthocyanidin monomeric units and polymers were highest in the fruit early in development and declined at stages T and R (Table 5.4), and the proanthocyanidin-specific

transcript studied in this study (LAR) was only detectable in high abundance at the earlier stages of development (Fig. 5.2), suggesting most proanthocyanidins were produced in early development. Proanthocyanidin synthesis in the early stages of fruit development also takes place rapidly in *F. x ananassa* (Carbone *et al*., 2006; Fait *et al*., 2009), blueberry (Zifkin *et al*., 2012) and red and yellow raspberry (Carvalho *et al*., 2013) with higher transcript abundance of LAR prior to ripening in *F. x ananassa* (Almeida *et al*., 2007, Carbone *et al*., 2009), and in red and white forms of *F. chiloensis* (Salvatierra *et al*., 2010), and *F. vesca* (Xu *et al*., 2014). The levels of catechin, epicatechin, and proanthocyanidin dimers in immature fruit and the lower content of each at R of white Pineapple Crush (Table 4) may have resulted from low expression of F3'H and DFR. In red *F. x ananassa* (Carbone *et al*., 2006; Thill *et al*., 2013) and *F. vesca* (Table 5; Xu *et al*., 2014b), an increase of transcript abundance of the anthocyanin-related genes ANS and UFGT accounted for the accumulation of anthocyanins. The low transcript level of ANS and UFGT at R in white *F. vesca* likely led to the absence of anthocyanin accumulation. White *F. chiloensis* fruit also showed down-regulation of the anthocyanin-related genes ANS and UFGT in comparison to a red form (Salvatierra *et al*., 2010). Lower expression of ANS was also reported in white fruited *Duchesnea indica* in contrast to a red fruited genotype (Debes *et al.*, 2011).

A product of the shikimate biosynthetic pathway, gallic acid, is integral to the production of derivatives of EA and ellagitannins. A higher content of EA derivatives and ellagitannins (Tables 5.5, 5.6) in white Pineapple Crush at the the T and R stages suggested that the lower flux of metabolites into the phenylpropanoid/flavonoid pathways resulting from reduced expression of PAL, CHS, F3'H, DFR, ANS and/or UFGT1 may increase metabolite flux into the shikimate pathway, increasing total ellagic/ellagitannin content.

Targeting individual phenylpropanoid/flavonoid pathway genes to reduce and/or eliminate expression has been shown to have similar effects on reduction of anthocyanin production and re-direction of some pathway intermediates as observed in white-fruited Pineapple Crush in this study. Silencing CHS, the branch point enzyme of the phenylpropanoid pathway, in *F. x ananassa* reduced anthocyanin content and increased 4-coumaroyl CoA derivatives (Lunkenbein *et al.*, 2006) and lignin (Ring *et al.*, 2013). RNAi-induced silencing of DFR in *F. x ananassa* resulted in an increase in quercetin derivatives, though only free quercetin was higher in the white form of *F. vesca* here. Also,

downregulation of F3H in the flavonoid pathway in *F. x ananassa* increased metabolites in the phenylpropanoid pathway.

In summary, this study provides a comprehensive examination of major differences in metabolite content in the phenylpropanoid/flavonoid biosynthetic pathway and related gene expression that exists in a red- and a white-fruited genotype of *F. vesca*. The phytochemical analyses of the distribution of specific phenolic compounds throughout fruit development of the white cultivar of *F. vesca* were performed for the first time. The intricate network of interactions between structural and regulatory genes with the production of particular metabolites are clearly separated in time from each other. Further studies will be needed to determine whether the changes in transcription factors and ABA-related genes regulate polyphenol and, specifically, anthocyanin synthesis in *F. vesca*. This study provides a general platform using naturally-occurring red and white strawberry for further investigation of this issue.

## Chapter 6: Effects of Phenolic Compounds on Growth of *Colletotrichum* spp. *in vitro*

### 6.1 Introduction

Anthracnose fruit rot is one of the most economically serious diseases of strawberry (*Fragaria x ananassa* Duch.) (Smith, 2008). The disease is primarily caused by *Colletotrichum acutatum* Simmonds, and affects fruit, flowers, leaves, petioles, crown and roots. Symptoms of strawberry anthracnose include blossom blight, defoliation, crown and root rot, and fruit rot. Two other *Colletotrichum* species, *C. gloeoesporoides* and *C. fragariae* Brooks, are also associated with fruit rot symptoms (Bailey *et al*., 1992). *Colletotrichum* spp. can cause pre-harvest disease on immature fruits in the field, and postharvest disease affecting mature fruits at harvest or during storage, depending on host specificity and environmental conditions (Wharton and Dieguez-Uribeondo, 2004). In general, fruit susceptibility is increased in association with physiological changes in fruit firmness, pH, cell wall composition, soluble sugars, and secondary metabolites that occur during fruit ripening (Sacher, 1973; Brady, 1987; Chillet *et al*., 2007; Moral *et al*., 2008). *Colletotrichum* spp. colonize unripe strawberry fruit via appressoria that are initially quiescent, whereas they penetrate ripe fruit directly via intercellular hyphae (Denoyes-Rothan *et al*., 1999; Curry *et al*., 2002; Guidarellia *et al*., 2011). The most effective way to control anthracnose fruit rot is to plant resistant cultivars, and there are a number of commercial strawberry cultivars that have some level of resistance to the pathogens (Seijo *et al.*, 2008). In susceptible cultivars, chemical control is possible but only with repeated and regular applications of fungicides (Wharton and Dieguez-Uribeondo, 2004; Mertely *et al*., 2004). Many of these synthetic fungicides are associated with carcinogenicity, teratogenicity, and residual toxicity to humans and other life forms, and have adverse effects on the soil ecosystem because they are non-biodegradable (Tegegne *et al*., 2008; Castillo *et al*., 2010). Repeated and regular applications can lead to another significant problem, the development of fungicide resistance within *Colletotrichum* spp. (Vincelli, 2002). Alternatives to synthetic fungicides would be highly desirable.

Plant-derived natural antifungal compounds may be an effective alternative to synthetic fungicides, either by creating host resistance within the plant, or via direct antifungal activity if applied exogenously. Phenolic and polyphenolic compounds are a potential source of natural antifungal compounds. These secondary metabolites can either

be preformed, acting as a natural chemical barrier to a pathogen, or can be synthesized during infection by a pathogen, as so-called phytoalexins (Treutter, 2006; Lattanzio *et al*., 2006). Strawberries have been established as a rich source of phenolic acids, flavonoids including proanthocyanidins and anthocyanins, and ellagitannins. Phenolic compounds can be toxic to an invading pathogen, with the free forms of phenolics considered more effective than their bound forms (Lattanzio *et al*., 2006). Flavan-3-ols have been found at significant levels in strawberry fruit (Buendia *et al*., 2010; Aaby *et al*., 2012; Del Bubba *et al*., 2012). In our studies of *Fragaria vesca*, high amounts of catechin and proanthocyanidin dimers were found at four developmental stages during fruit development and ripening. Proanthocyanidins have been reported as infection-inhibiting factors against *Botrytis cinerea* (gray mold) infection of commercial strawberry (Treutter *et al*., 1991; Yamamoto *et al*., 2000). Conjugates of ellagic acid, epicatechin, proanthocyanidins, hydroxycinnamic acid derivatives, and some flavonols increased in strawberry fruit during infection by *C. nymphaeae* (Milkulic-Petkovsek *et al*., 2013). Infection of citrus peel (*Citrus benikoji*) by *C. gloeosporiodes* promoted a gradual increase in the content of seven flavonoids and produced the de novo phytoalexin, hespertin-7-*O*-glucoside (Jeong *et al*., 2014). Gogoi *et al*. (2001) reported a rapid accumulation of phenols at the site of a fungal infection of wheat (*Triticum* spp.) that inhibited or restricted pathogen growth. The effect of some phenolic acids and flavonoids against several fungal species *in vitro*, including *Aspergillus* spp. (Chipley and Uraih, 1980; Nesci and Etcheverry, 2006), *Penicillium* spp. (Florianowicz *et al.,*1998), *Botryodiplodia theobromal* (Mohapotra *et al*., 2000), and *Fusarium oxysporum*, *Sclerotinia sclerotiorum,* and *Phlyctaena vagabonda* (Lattanzio *et al.,* 2001) has been documented. However, there are few reports of the direct effect of these polyphenols on the growth of *Colletotrichum* spp. Thus, the hypothesis for this study was that some but not all phenolic compounds found in strawberry would inhibit the growth of *Colletotrichum* spp. *in vitro*. The approach of this study was to evaluate the efficacy of several free forms of phenolic compounds found in strawberry for their ability to inhibit growth of *Colletotrichum* spp. that cause strawberry anthracnose *in vitro.*

## 6.2 Material and Methods

### 6.2.1 Fungal culture

*Colletotrichum* spp. isolates used in this study and their origins are listed in Table 1. All strains were described by Du *et al.*, (2005). Cultures were maintained on potato dextrose agar (PDA, Difco) in 9 cm diameter Petri dishes, and were grown at 23$^{o}$ C under cool white fluorescent lights for 14 d before use.

### 6.2.2 Chemicals and solvent

Phenolic acids (gallic acid, caffeic acid, chlorogenic acid, ferulic acid, *trans*-cinnamic acid, *p*-coumaric acid, salicylic acid) and flavonoids (catechin, quercetin) were chosen for this study based on their reported presence in *Fragaria* spp., cost, and availability. All compounds were obtained from Sigma-Aldrich (St Louis, MO). Each standard was dissolved in 100% methanol at a stock concentration of 0.1 M and stored at 4$^{o}$C until used. For the assays, stock solutions were diluted with 100% methanol to a final concentration of 5, 10, and 50 mM. Difco PDA (Becton, Dickinson and Company, Sparks, MD). The fungicide Pristine (pyraclostrobin; BASF Corporation, Research Triangle Park, NC) was used at 1.7 g/L as a positive control, following manufacturer's instructions.

### 6.2.3 Well-plate toxicity assay

Two-week-old fungal cultures were flooded with 2-3 mL of sterile water, and the fungal mat was scraped gently with a sterile pestle. The resulting spore suspension was filtered through sterile cheesecloth into a 50 mL centrifuge tube, and the volume was brought to 40-45 mL by adding sterile water. Spores were pelleted by centrifugation at 1600 x g for 10 min. After decanting the supernatant, the spores were washed twice in 40-45 sterile water, followed by centrifugation at 1600 x g for 10 min. Finally, the spores were resuspended in 20-30 mL of sterile water and added to 3 ml of molten 0.6% agarose at 45°C to produce a final concentration of 1 x $10^4$ or 5 x $10^4$ conidia·mL$^{-1}$. The spore-agar solution was poured evenly onto the surface of a plate containing 30-33 mL of solidified PDA to create a uniform layer.

After allowing the agarose top layer to solidify for 3 h, four wells were made at evenly spaced intervals in each dish with a No. 4 corkborer. Then, 0.1 mL of a standard

phenolic or flavonoid solution was pipetted into each well. Three concentrations, 5, 10 and 50 mM, of each phenolic or flavonoid standard were used in each dish, along with 100% methanol as the control. Each dish was replicated three times. The plates were incubated at 23°C for 36 h in the dark. Antifungal activity was expressed as the diameter of the zone of visible inhibition of growth of the fungal lawn, minus the diameter of the well (Kerr *et al*., 1999).

### 6.2.4 Statistical analyses

The data was statistically analyzed for linear and quadratic trends across concentrations within spore density and compound, and for comparison of the initial spore density

**Table 6.1** list of the *Colletotrichum* spp. isolates used in this study.

| **Species** | **Isolate** | **Host** | **Origin** |
|---|---|---|---|
| *C. acutatum* | Goff99 | strawberry | Missouri |
| *C. acutatum* | Mil1 | strawberry | Missouri |
| *C. acutatum* | 2.6.23 | strawberry | Arkansas |
| *C. acutatum* | 2.7.15 | strawberry | New Zealand |
| *C. acutatum* | TUT137A | strawberry | Israel |
| *C. acutatum* | APPY3 | apple | Kentucky |
| *C. gloeosporioides* | FA16 | strawberry | Arkansas |
| *C. gloeosporioides* | FC216 | strawberry | Arkansas |
| *C. fragariae* | CF75 | strawberry | Michigan |
| *C. graminicola* | M1.001BH | corn | Missouri |

within each compound, and the compounds were compared at only the maximum concentration (50 mM) within spore density levels by ANOVA with mean separation by Fisher's least significant difference at $P<0.05$ using SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA).

### 6.3 Results and Discussion

Of the compounds tested, only *trans*-cinnamic acid, ferulic acid and *p*-coumaric acid inhibited fungal growth (Fig 6.1). The 100% methanol control, and the highest concentrations of gallic acid, chlorogenic acid, caffeic acid, salicylic acid, naringenin, quercetin, catechin, and ellagic acid tested, 50 mM, exhibited no obvious inhibitory effect on growth of any of the isolates of *Colletotrichum* spp. in this study.

The *Colletotrichum* isolates were inhibited in significant linear and quadratic patterns ($P<0.05$) with increasing concentrations of *trans*-cinnamic acid, ferulic acid and *p*-coumaric acid within each initial spore suspension concentration (Figs. 6.2-6.4). At 5 mM *p*-coumaric acid, *C. gloeoesporiodes* FA16 was not inhibited at either spore concentration, and at the higher 5 x $10^4$ conidia·mL$^{-1}$ suspension, Goff99 and TUT137A (*C. acutatum),* FA16 and FC216 (*C. gloeosporioides*), and CF75 (*C. fragariae*) were not inhibited. So, in the latter case, higher concentrations of spores were great enough to overcome potential inhibition by the low level of *p*-coumaric acid.

Responses among isolates and between compounds within each spore suspension concentration were compared at 50 mM (Table 6.2). The isolates did not display the same responses at the different spore suspension concentrations. Ferulic acid exhibited the highest inhibitory activity (i.e., largest inhibitory zone) against FC216 at 1 x $10^4$ and 5 x $10^4$, but Goff99 was equally inhibited at only 5 x $10^4$. Isolate 2.6.23 was least inhibited by ferulic acid at 1 x$10^4$, but Mil1 was least inhibited at 5 x$10^4$. *p*-Coumaric acid had limited activity against 2.6.23 and Goff99 at 1 x $10^4$ and 5 x $10^4$, respectively. *trans*-Cinnamic acid was most inhibitory against M1.001BH at both spore suspension concentrations, and least effective against Mil1, 2.6.23, and CF75 at 1 x $10^4$, and only Mil1 at 5 x $10^4$. Within each isolate, the greatest inhibitory activity was shown by *trans*-cinnamic acid followed by ferulic acid, then *p*-coumaric acid, irrespective of spore suspension concentration.

The results indicated that *trans*-cinnamic acid had the greatest inhibitory effect on all *Colletotrichum* isolates tested. Although the three compounds are very similar in structure,

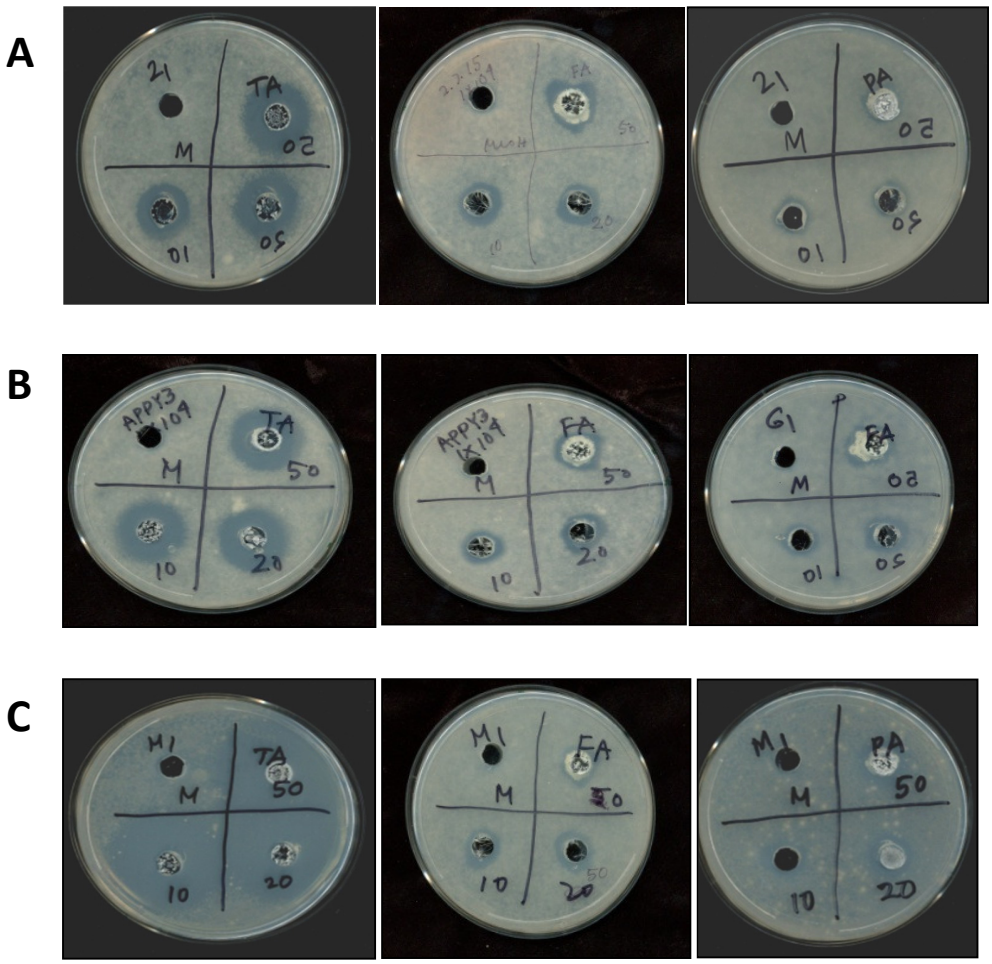



**Figure 6.1** Well-plate toxicity dishes with *Colletotrichum* isolates. A) 2.7.15, B) APPY3, and C) M1.001BH with *trans*-cinnamic acid, ferulic acid and *p*-coumaric acid (from left to right).

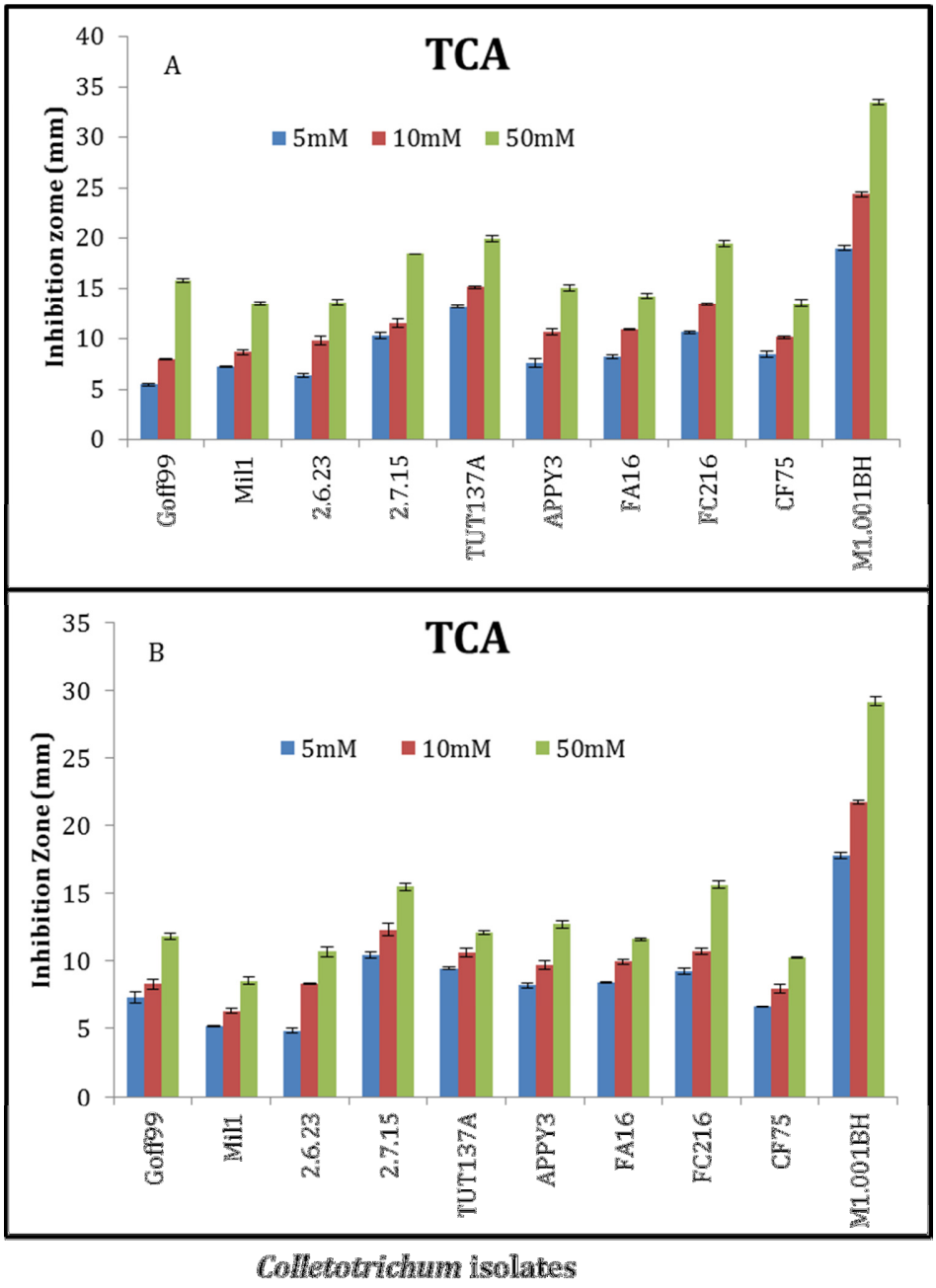



**Figure 6.2** Effect of *trans*-Cinnamic acid (TCA) on inhibition of growth of *Colletotrichum* spp. at 5, 10, 50 mM.

Mean values ± SD (n=3) represent the diameter of the zone of inhibition. The control solution was 100% MeOH and resulted in no (or zero) inhibition. A) Spore suspension at 1 x $10^4$ conidia $mL^{-1}$. B) Spore suspension at 5 x $10^4$ conidia $mL^{-1}$. There were significant linear and quadratic trends across concentration within each isolate at $P<0.05$.

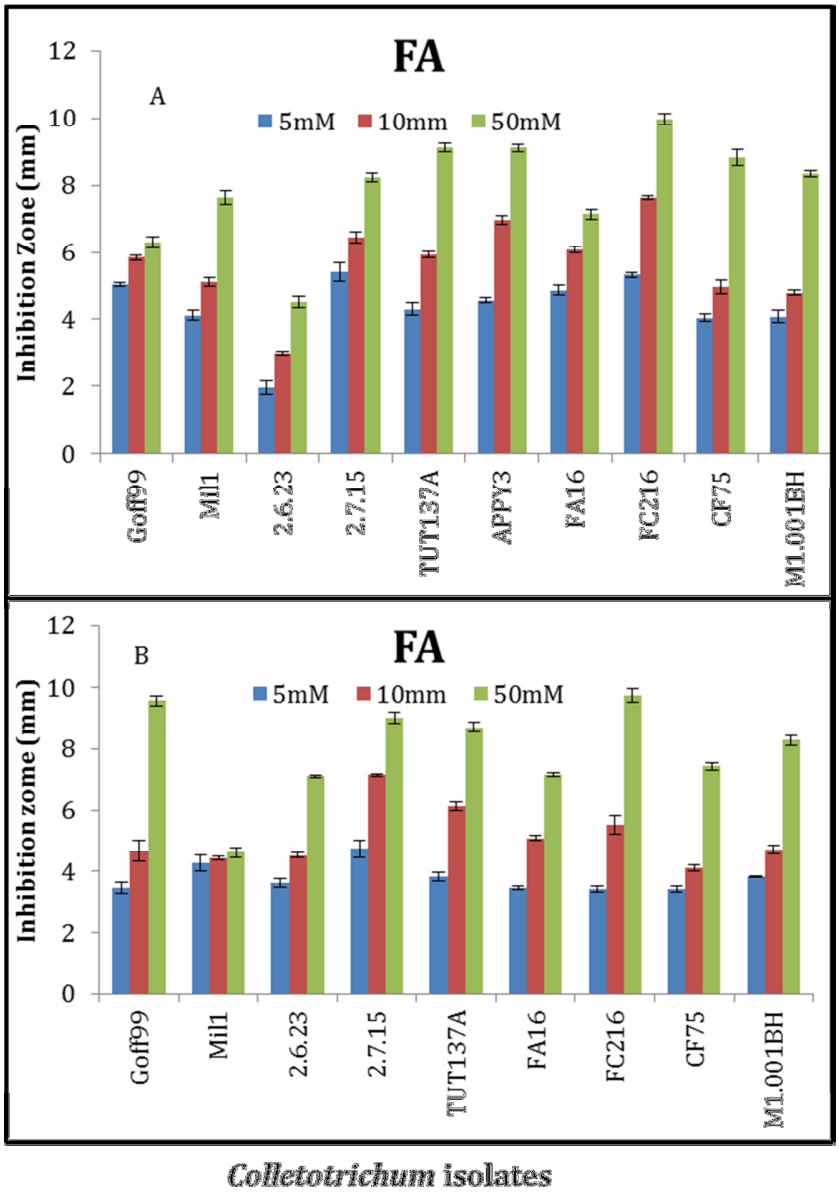



**Figure 6.3** Effect of ferulic acid (FA) on inhibition of growth of *Colletotrichum* spp. at 5, 10 and 50 mM.

Mean values ± SD (n=3) represent the diameter of the zone of inhibition. The control solution was 100% MeOH and resulted in no (or zero) inhibition. A) Spore suspension at 1 x $10^4$ conidia $mL^{-1}$. B) Spore suspension at 5 x $10^4$ conidia $mL^{-1}$. There were significant linear and quadratic trends across concentration within each isolate at $P<0.05$.

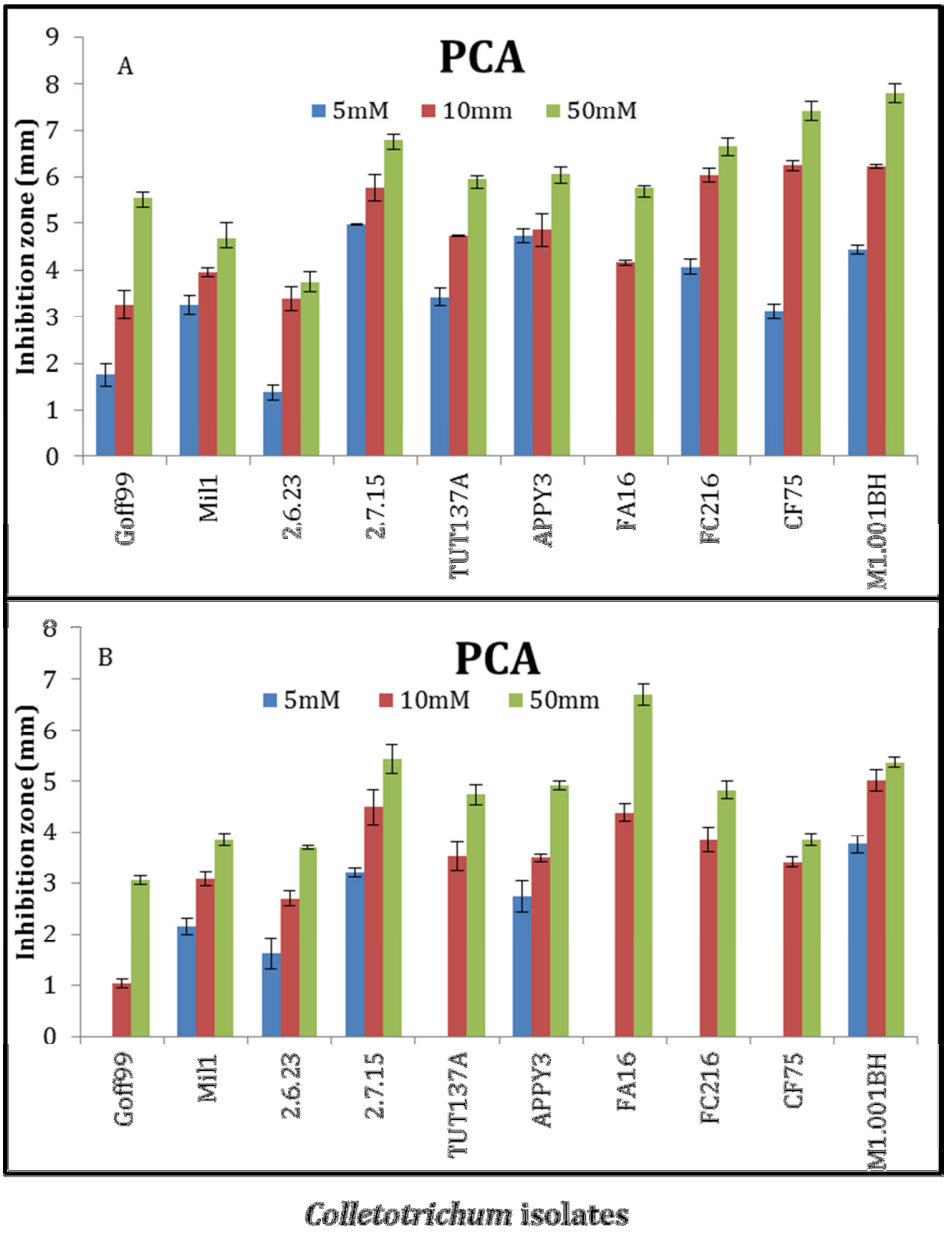



**Figure 6.4** Effect of *p*-Coumaric acid (PCA) on inhibition of growth of *Colletotrichum* spp. at 5, 10 and 50 mM. Mean values ± SD (n=3) represent the diameter of the zone of inhibition. The control solution was 100% MeOH and resulted in no (or zero) inhibition. A) Spore suspension at 1 x $10^4$ conidia $mL^{-1}$. B) Spore suspension at 5 x $10^4$ conidia $mL^{-1}$. There were significant linear and quadratic trends across concentration within each isolate at $P<0.05$.

**Table 6.2** Effect of 50mM ferulic acid (FA), p-Coumaric acid (PCA) and trans-Cinnamic acid (TCA) on inhibition of mycelial growth of Colletotrichum spp. isolates st initial spore suspension of 1 x $10^4$ conidia $mL^{-1}$ and 5x $10^4$ conidia $mL^{-1}$. Mean values represent the diameter of the zone of inhibition. The control solution was 100% MeOH and resulted in no (or zero) inhibition.

| | 1 x $10^4$ conidia·$mL^{-1}$ | | | 5 x $10^4$ conidia·$mL^{-1}$ | | |
|---|---|---|---|---|---|---|
| **Isolate** | **FA** | **PCA** | **TCA** | **FA** | **PCA** | **TCA** |
| Goff | 6.3 G b[z] | 5.6 E c | 15.8 E a | 9.8 A b | 3.1 E c | 11.8 EF a |
| *Mil1* | 7.6 E b | 4.7 F c | 13.4 H a | 4.6 G b | 3.8 D c | 8.6 H a |
| *2.6.23* | 4.5 H b | 3.7 G c | 13.5 H a | 7.1 F b | 3.7 D c | 10.7 G a |
| *2.7.15* | 8.2 D b | 6.8 C c | 18.4 D a | 9.0 B b | 5.4 B c | 15.5 B a |
| *TUT137A* | 9.1 B b | 6.0 D c | 19.9 B a | 8.8 B b | 4.7 C c | 12.0 E a |
| *APPY3* | 9.1 B b | 6.1 D c | 15.0 F a | 7.8 D b | 4.9 C c | 12.7 D a |
| *FA16* | 7.1 F b | 5.8 DE c | 14.2 G a | 7.3 E b | 6.7 A c | 11.6 F a |
| *FC216* | 10.0 A b | 6.6 C c | 19.4 C a | 9.8 A b | 4.8 C c | 15.6 B a |
| *CF75* | 8.8 C b | 7.4 B c | 13.5 H a | 7.4 EF b | 3.8 D c | 10.3 G a |
| *M1.001BH* | 8.3 D b | 7.8 A c | 33.4 A a | 8.3 C b | 5.4 B c | 29.2 A a |

[z]Mean separation among isolates within spore concentration and phenolic acid by Fisher's LSD at P<0.05 shown by upper case letters, and among phenolic acids within isolates and spore concentration by Fisher's LSD at P<0.05 shown by lower case letters.

differences in their diffusion rates in the media could lead to the same conclusion, i.e., greater diffusion led to greater apparent effect. Because there are no reports about possible inhibitory effects of phenolic compounds on *Colletotrichum* spp., the present results were compared to data available for other fungal pathogens. Derivatives of benzoic and *trans*-cinnamic acid inhibited growth of *Aspergillus* and *Penicillium* spp. (Chipley and Uriah, 1980; Florianowicz *et al.,* 1998; Nesci and Etcheverry, 2006). The effect of derivatives of benzoic acid and *trans*-cinnamic acid at 0.2 mg/mL were inhibitory on the growth and release of aflatoxin by *A. parasiticus* (Chipley and Uraih, 1980). Nesci and Etcheverry (2006) reported that 10-20 mM *trans*-cinnamic acid was effective at inhibiting growth of four species of *Aspergillus*. Similar to our results, *trans*-cinnamic acid at 10 mM inhibited *Aspergillus flavus* (Kim *et al.,* 2004), whereas *o*-coumaric, *m*-coumaric and *p*-coumaric inhibited hyphal growth from 5 to 25 mM, and caffeic acid had limited antifungal activity even above 25 mM. Fungal growth of *Fusarium oxysporum* and *Sclerotinia sclerotiorum* were inhibited by ferulic acid (Lattanzio *et al.*, 1996) In contrast to our results, cholorogenic acid exhibited the highest inhibition of *Botryodiplodia theobromal in vitro* (Mohapotra *et al.*, 2000), and also inhibited *Phlyctaena vagabonda* (Lattanzio *et al*., 2001). *Phlyctaena vagabonda* was not inhibited by (+) catechin or quercetin glycosides (Lattanzio *et al.,* 2001), similar to our results.

Phenolic compounds are produced by species throughout the plant kingdom, but there is significant variability in their presence and content among species, which could affect the response to attack by a wide range of potential pathogens (Lattanzio *et al*., 2006). The number and position of the hydroxyl groups on the aromatic ring of phenolic acids influence the antioxidant capacity of the compound (Sekhar *et al.*, 2001). The defensive strategies of plants involve producing phenolic compounds during development, and rapid accumulation of phenols an infection site which may slow down or inhibit the growth of the pathogen, often followed by de nevo synthesis of phytoalexins (Mansfield, 1982; Matern and Kneusel, 1988; Nicholson and Hammerschmidt, 1992; Wharton and Nicholson, 2000). Resistant onion varieties for onion smudge disease showed lower spore germination of *Colletotrichum circinas* for the presence of sufficient amounts of preformed catechol and procatechuic acid (Walker and Stahmann, 1955). The accumulation of phenolic compounds are so rapid that there are non-biosynthetic means other than sequential de novo transcription and translation of genes associated with synthhesis of phenolic compounds as a primary defense response (Nicholson and Hammerschmidt, 1992). Enzymatic

modifications may be initiated with a substantial decrease in cytoplasmic pH (Kneusel *et al*., 1989), activating a hydroxylase to convert 4-coumaroyl CoA to caffeoyl CoA, for example. Potentially toxic ferulic acid can be synthesized by *S*-adenosyl L-methionine *trans*-caffeoyl coenzyme A 3-*O* methyltransferase (Pakusch *et al*., 1989; Nicholson and Hammerschmidt, 1992). The concentration of phytoalexins within a single host cell can greatly exceed that which is necessary for the *in vitro* inhibition of the fungus (Nicholson and Hammerschmidt, 1992; Wharton and Nicholson, 2000).

The present results indicated that antifungal activity of particular polyphenols may vary depending on the specific fungal organism. Several species of *Colletotrichum* cause strawberry anthracnose (Howard *et al*., 1992), and strawberry fruit have a wide array of polyphenols. Cholorogenic acid, caffeic acids, *p*-couramic acid and ferulic acids were the most abundant hydroxycinnamic acids in *F. x ananassa* found in our previous study (Chapter 4), and has been reported by others (Buendia *et al*., 2010; Aaby *et al*., 2012). Overall, the concentrations of these phenolic acids are lower in strawberry in comparison to other phenolics and flavonoids. In our study, total hydroxycinnamic acid content was 0.082 mg/100 g FW in one cultivar of *F. x ananassa.* Derivatives of coumaric acid ranged from 0.5-0.9 mg/100 g FW in our study and that of others (Buendia *et al*., 2010; Kelebek *et al*., 2011). Other major flavonoids, quercetin derivatives, and flavan-3-ol derivatives exhibited considerably higher amounts in ripe fruit in comparison to the phenolic acids. Major flavan-3-ols, catechin, and procyanidin derivatives were 1.8-10.6 mg/100 g FW in our study with others reporting a similar or slightly higher concentration depending on the cultivar of *F. x ananassa (*Buendia *et al.,* 2010; Kelebek *et al*., 2011; Aaby *et al*., 2012). Our *in vitro* results indicated the possibility of no or minimal effect of these compounds on resistance to *Colletotrichum* in strawberry. However, apart from the present study, the inhibitory effects of the individual preformed phenolic compounds from strawberry with *Colletotrichum* have not been reported.

Possible synergistic effects of the compounds tested in this work may be possible, as strawberry contains many of them, but this was not assessed in the present study. This is supported by the fact that there are changes in secondary metabolite content and composition in response to the fungal inoculation in strawberry which suggest that synergistic effects may provide some fungal inhibition. Increased amounts of ellagic acid conjugates, quercetin-3-glucoronide, epicatechin, and oligomeric procyanidins were

reported in two *Colletotrichum*-infected cultivars of *F. x ananassa* (Milkulic-Petkovsek *et al*., 2013). Powdery mildew (*Sphaerotheca macularis*) infection of strawberry caused a higher accumulation of *p*-coumaroyl glucoside (Hukkanen *et al*., 2007; Milkulic-Petkovsek *et al*., 2009, 2013), though no clear data on the mechanism of the interaction are available. More research needs to be done to investigate the effect of the individual as well as collective contribution of phenolic compounds to strawberry-*Colletotrichum* spp. pathogen interactions.

## Chapter 7: Summary and Conclusions

The primary objective of this research was to determine if there are differences in polyphenolic content among natural white-fruited mutants of *F. vesca* indicative of different mutations, knowledge of which could contribute to an understanding of metabolic flux through the complex, branched phenylpropanoid/flavonoid biosynthetic pathways during fruit development. Thus, selected phenolic-derived compounds in ripe berries of several genotypes were identified and targeted as representatives of major groups in the biosynthetic pathways for comparison, and the evolution of these compounds during berry development were studied at the biochemical and molecular levels. The implications of such changes in the polyphenolic profile were explored by studying the potential contribution of selected phenolic compounds to *Colletotrichum* spp. disease resistance.

Quantification of the polyphenols in red- and white-fruited cultivars of *F. vesca* and *F. x ananassa* yielded different results between UV spectrophotometric and HPLC-MS analyses, with the former indicating generally higher values. Total phenolics and flavonoids were more in white cultivars of *F. vesca* assays (Table 3.1) whereas the opposite was observed using targeted compounds in HPLC-MS (Figure 4.3). The proanthocyanidin levels were lower for white *F. vesca* in both the studies (Tables 3.1, 4.5) The cultivars of *F. x ananassa* showed a similar pattern of lower levels of polyphenols in white White Pine than the red Earliglow in both the studies (Table 3.1, Fig. 4.3). Anthocyanins were not detected in white cultivars of *F. vesca* and *F. x ananassa* by UV spectrophotometry (Table 3.1), but very low levels of cyanidin and pelargonidin derivatives were detected in these genotypes by HPLC-MS (Tables 4.2, 4.3; Fig. 4.1).

Spectrophotometric methods provide a general quantification of total content of classes of compounds, but they also have problems which reduce their reliability. Non-phenolic compounds with similar chemical properties can interfere with the assays (Robards and Antolovich, 1997). Variable specificity and detection sensitivity of the reagents for specific phenolics can also compromise quantitative detection (Robards and Antolovich, 1997; Ignat *et al*., 2011; Pękal and Pyrzynska, 2014). HPLC-MS analyses provide more reliable quantification of complex and structurally diverse polyphenols with different molecular weights via their separation and specific detection.

Selected polyphenols, 21 total, from ripe berries of several *F. vesca* and *F. x ananassa* cultivars were identified and quantified by HPLC-MS, and used as representatives of the

major polyphenolic classes in the *Fragaria* spp. Total anthocyanins and non-anthocyanin polyphenols (flavonols, free and conjugated hydroxycinnamic acids and proanthocyanidins) were significantly reduced in the white mutants of both species, suggesting a general reduction in phenylpropanoid enzyme activities. However, polyphenolic content differed among some of the white *F. vesca* suggesting independently-derived mutation(s) responsible for their differences. The ellagic acid derivatives and ellagitannin content varied among white *F. vesca* cultivars, but three of the white genotypes had a higher total EA/ET content than red genotypes, suggesting the possibility of a shift in carbon flux to the EA/ET pool in these genotypes. Similar patterns of accumulation of polyphenols were found during fruit development of a white-fruited *F. vesca* cultivar (Fig. 4.3, Tables 4.7, 5.7). Though there was a decline in EA/ET content in both red and white-fruited *F. vesca* during fruit development, the content seemed to stabilize by the turning stage in a white genotype while it continued to drop in the red genotype.

Expression analyses of the structural genes of the flavonoid biosynthetic pathway indicated a correlation between transcript abundance of the genes and the accumulation of related polyphenolic end products. The lower content of proanthocyanidins and the absence of anthocyanins in a white genotype at the T and R stages suggested that the lower flux of metabolites into the phenylpropanoid/flavonoid pathways resulted from reduced expression of PAL, CHS, F3'H, DFR, ANS and/or UFGT1. Lower abundance of the transcription factor MYB10 in the later stages of fruit development of the white genotype support its key role in regulation of the anthocyanin related gene UFGT1 (Medina-Puche *et al*., 2014; Lin-Wang *et al*., 2014). However, further clarification of regulation is needed as neither the expression of the transcription factor MYB1, the ABA biosynthetic gene NCED1 or the ABA receptor gene ABAR/CHLH were related to lack of color development as others have reported for *F. x ananassa* (Medina-Puche *et al*., 2014), suggesting the possibility that the regulation of the phenylpropanoid/flavonoid metabolic pathway varies among *Fragaria* species (Winkel-Shirley, 2001).

The efficacy of nine phenolic acids and flavonoids found in *Fragaria* spp., tested in well-plate toxicity assays on ten *Colletotrichum* spp. isolates, indicated that only three were antifungal, *trans*-cinnamic acid, ferulic acid, and *p*-coumaric acid. Although these three compounds have been found in *Fragaria* spp., they were at much lower levels than was effective *in vitro.* Nonetheless, they may provide a path that could be exploited. Transgenic

plants that could over-produce the phenolic compounds could be tested to compare their *in vitro* and *in vivo* effects against *Colletotrichum* isolates.

The availability of Agrobacterium-mediated transformation protocols, loss-of-function mutant collections and protocols for large scale metabolite syntheses are increasing for *Fragaria* spp. (Oosumi *et al.*, 2010; Shulaev *et al*., 2011; Aaby *et al*., 2012). Research with the model species *F. vesca* to understand the complex and interconnected biosynthetic pathways leading to the synthesis of diverse polyphenols is only recent for the most part. Further studies to analyze the impact of overexpression of MYB10 in white-fruited *F. vesca* may confirm its role suggested from studies of red *F. x ananassa*. In a recent study, Schaart *et al.* (2013) reported that two regulatory genes, FaMYB9 and FaMYB11, interacted with FaTTG1 to regulate PA accumulation during early stages of fruit development in *F. x ananassa.* These TFs would be interesting candidates for studies in white *F. vesca* due to its lack of anthocyanin.

The content of proanthocyanidins and ellagic acid derivatives and ellagitannins in ripe *F. vesca* and *F. x ananassa* fruit, along with their accumulation patterns during fruit development arouse questions about the enzyme activities in these individual subnetworks that determine the final content of the end products. Although beyond the scope of the present work, studies of key enzyme activities in the red- and white-fruited *F. vesca* genotypes during fruit development would shed further light on how the two genotypes differ.

The shikimate pathway has not yet been studied in detail in *F. vesca*. Given the possibility of a shift to EA/ET products in some of the white *F. vesca* genotypes, quantification of key intermediates and gene expression leading to the branch point of both pathways including phosphoenol pyruvate, erythrose-4-phosphate, deoxy-D-arabino–heptulosonic acid-7-phosphate (DHAP), and of the enzyme DHAP synthase, leading to chorismate which is responsible for the production of the precursor for the phenylpropanoid pathway, phenylalanine, and to key steps in EA/ET production by shikimate dehydrogenase protocatechuic acid hydroxylase, could shed further light on how the genotypes differ.

# Appendix A: Differentiating Strawberry Genotypes by Principal Component Analysis and Hierarchical Cluster Analysis

## A.1 Introduction

The goal of the present study was to differentiate among eight red and white genotypes of *F. vesca* and *F*. x *ananassa* based on their polyphenolic profiles, presented in Chapter 4. Principal component analysis (PCA) is a useful statistical tool with which to perform a reduction in data dimensionality and allow visualization of experimental data. This permits a relatively simple projection of relationships between samples from complex analytical data (Hossain *et al*., 2011). Hierarchical clustering analysis (HCA) can be used as an alternative approach to understand common patterns in complex data sets by clustering genotypes by their traits into categories (Endo *et al*., 1990). Because the hypothesis of this study was that there would be differences between red and white genotypes of *F. vesca* and *F*. x *ananassa*, and especially among some of the white genotypes of *F. vesca*, shown by the results of the analyses of variance with mean separation, PCA and HCA analyses were used to explore this further.

## A.2 Materials and Methods

Principal components analysis (PCA) was calculated with the phenylpropanoid/flavonoid data in Chapter 4 using the 'princomp' function of S-Plus (MathSoft Inc.). Hierarchical clustering analysis was performed using MEV (Multi-experiment viewer) version 4.9 software. The analyses were provided by Dr. Benhong Wu and Wenwen Liu, Ph.D. candidate, of the Institute of Botany, Chinese Academy of Sciences, Beijing, China.

## A.3 Results and Discussion

The PCA sample scores for PC1 and PC2 were 52.5% and 20.0%, respectively, a total of 72.5%, of total variance (Figure A.1). Comparing the genotypes, red Earliglow showed the highest positive loadings in both PC1 and PC2 axes. The other red genotype, Baron

Solemacher, showed a positive loading for PC1 but the lowest negative loading in PC2. White Pine, the white *F. X ananassa*, had nearly neutral (close to 0) loadings in both PC1 and PC2. The white *F. vesca* genotypes Ivory, Yellow Wonder, Baron Solemacher White, and Pineapple Crush had negative

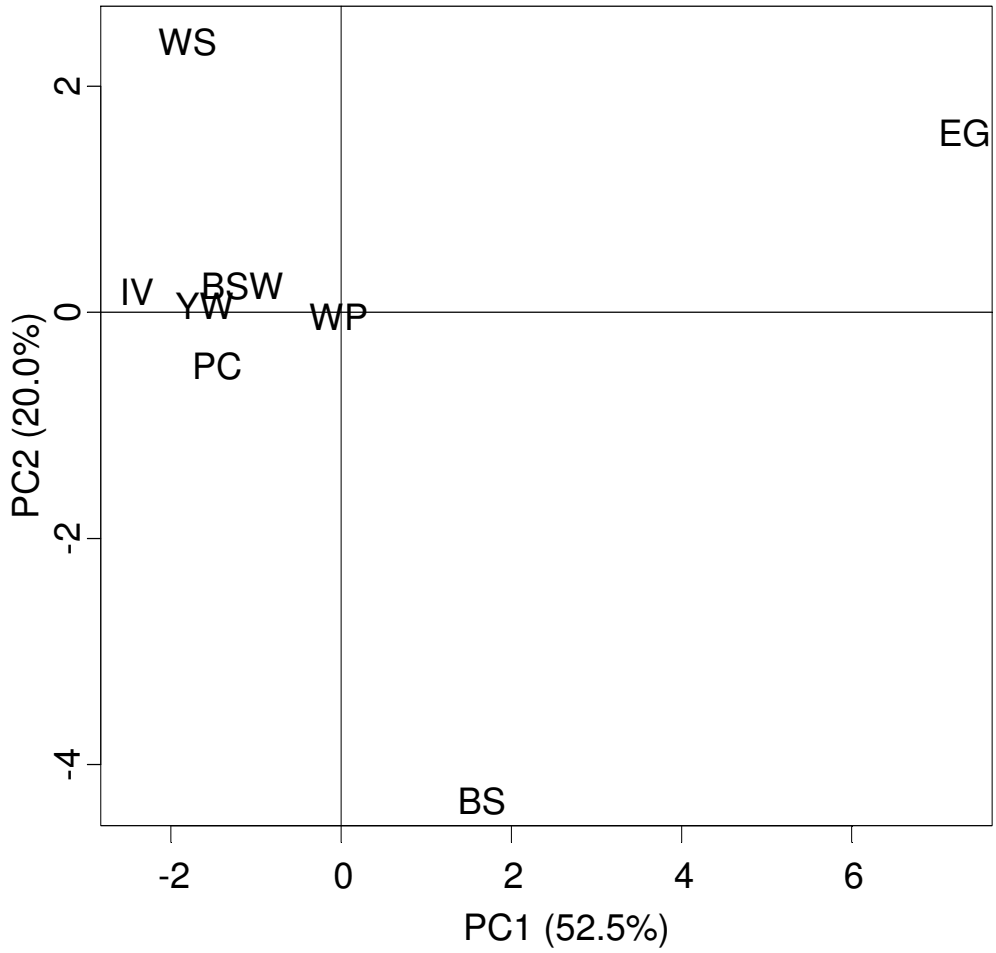



**Figure A.1** Principal component analysis (PCA) of genotypes. *F. vesca* genotypes red Baron Solemacher (BS), and white Baron Solemacher White (BSW), Ivory (I), Pineapple Crush (PC), White Soul (WS), and Yellow Wonder (YW), and the *F.* X *ananassa genotypes* red Earliglow (EG) and white White Pine (WP) based on phenylpropanoids/flavonoid metabolites. Genotype scores are for the first two PCs. Percentages in brackets represent the variance of each component.

loadings in PC1. In PC2, Ivory, Yellow Wonder, and Baron Solemacher White were nearly neutral (close to 0). One white *F. vesca* genotype, White Soul, was separate from the other white *F. vesca* genotypes with a negative PC1 value and the highest positive PC2 value. The genotypes Earliglow, Baron Solemacher and White Soul were the most distinctly unique genotypes, and Pineapple Crush and Ivory also appeared to differ some from the other white *F. vesca*.

Examination of the metabolite loadings within PC1 and PC2 axes revealed compounds in all quadrants of the two-dimensional PCA plots (Figure A.2). Negative loadings in PC1 resulted in separation of quercetin, caffeic acid and kaempferol coumaroyl hexoside from all of the other metabolites. Most of the anthocyanin derivatives and flavan-3-ols showed positive PC1 loadings and negative PC2 loadings. Most of the flavonols and hydroxycinnamic acids showed positive loadings in both PC1 and PC2 axes. The exceptions were the flavonol kaempferol coumaroyl hexose and the hydroxycinnamic acid caffeic acid with negative loadings on PC1 and positive loadings on PC2. The positive loading of red cultivars of *F. vesca* and *F* x *ananassa* in PC1 (Figure A.1) corresponded to the positive loading of anthocyanin derivatives and proanthocyanidins, indicating the high content of these compounds in these two cultivars and differentiating them from the negative loading of all of the white cultivars on the PC1 axis (Figure A.1). The high negative loading of the red Baron Solemacher (Figure A.1) corresponded to the negative loading of catechin, epicatechin, proanthocyanidin dimers, cyanidin derivatives, quercetin and quercetin-3-glucoside on the PC2 axis (Figure A.2) . Among white-fruited *F. vesca* genotypes, all (Figure A.1) corresponded to the negative loadings of quercetin, caffeic acid, and kaempferol coumaroyl hexose on the PC1 axis (Figure A.2). However, Pineapple Crush was the only one with a negative loading on the PC2 axis (Figure A.1), corresponding to the negative loading of the flavan-3-ols (Figure A.2). In contrast, the other *F. vesca* genotypes had positive loadings on the PC2 axis (Figure A.1) corresponding to the positive loadings of the flavonols and hydroxycinnamic acids (Figure A.2).

A combined heatmap and hierarchical cluster analysis of phenylpropanoid/flavonoid metabolites showed that the most abundant flavonoids in all genotypes were proanthocyanidin dimers and catechin (Figure A.3). The abundance of anthocyanin derivatives in the red genotypes of *F. vesca* and *F* x *ananassa* were a primary cause of their difference from the white genotypes. The HCA indicated two main clusters of phenylpropanoids/flavonoid metabolites, one showing a lower level of chlorogenic acid,

caffeic acid, *p*-coumaric acid, quercetin and kaempferol coumaroyl hexoside, and the other contained several metabolites at higher levels. The HCA of the genotypes clearly showed the red Earliglow (*F.* x *ananassa*) and Baron Solemacher (*F. vesca*) clustered together, the former with higher amounts of cholorgenic acid, quercetin-3-glucuronide, kaempferol-

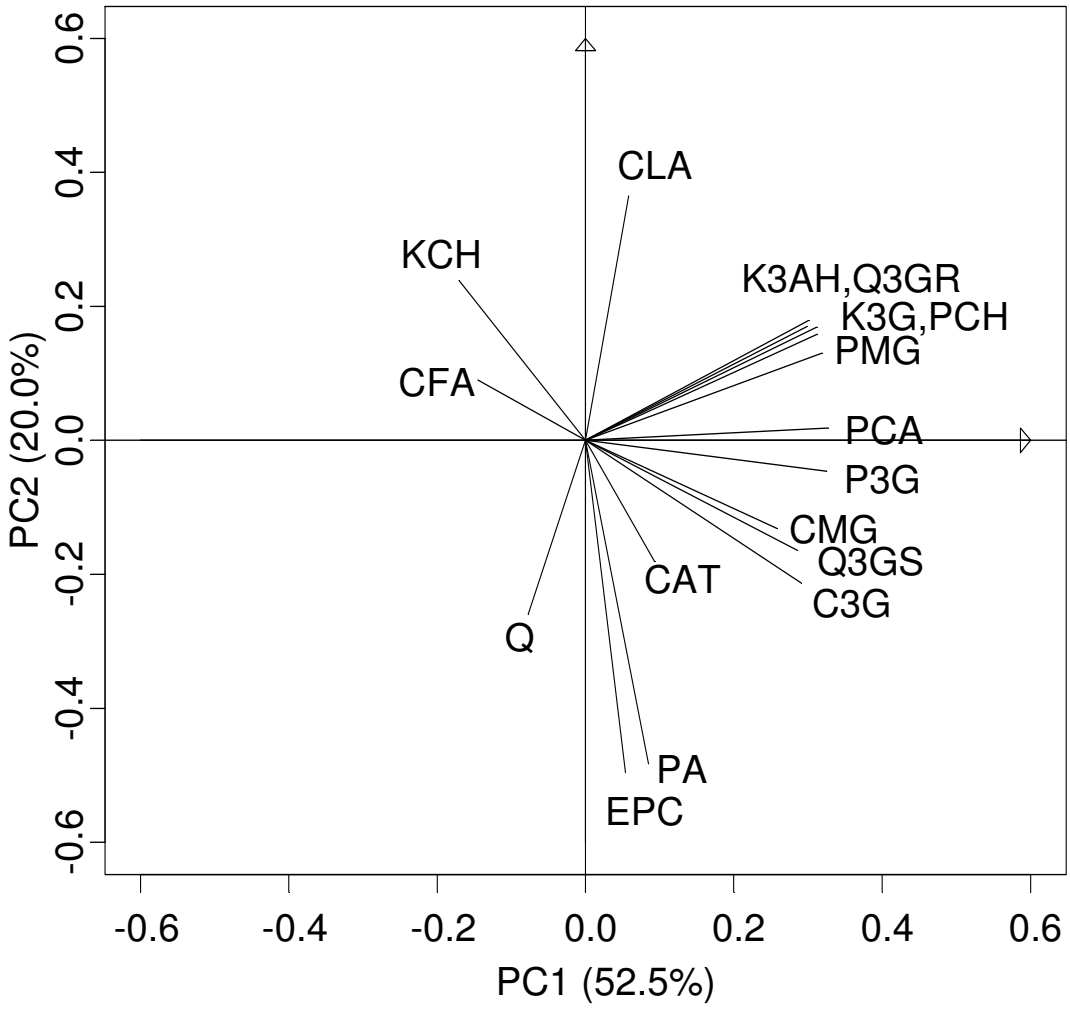



**Figure A.2** Principal component analysis of the phenylpropanoid/flavonoid metabolites:

(Q), quercetin-3-glucoside (Q3GS), quercetin-3-glucuronide (Q3GR), kaempferol-3-glucoside (K3G), kaempferol coumaroyl hexose (KCH), kaempferol malonylglucoside (KMG), kaempferol-3-acetyl hexose (K3AH), caffeic acid (CFA), chlorogenic acid (CLA), *p*-coumaric acid (PCA), epicatechin (EPC), catechin (CAT), proanthocyanidin dimers (PA), pelargonidin-3-glucoside (P3G), pelargonidin malonylglucoside (PMG), cyanidin-3-glucoside (C3G), and cyanidin malonylglucoside (CMG). Loadings of flavonoids are for the first two PCs. Percentages in brackets represent the variance of each component.

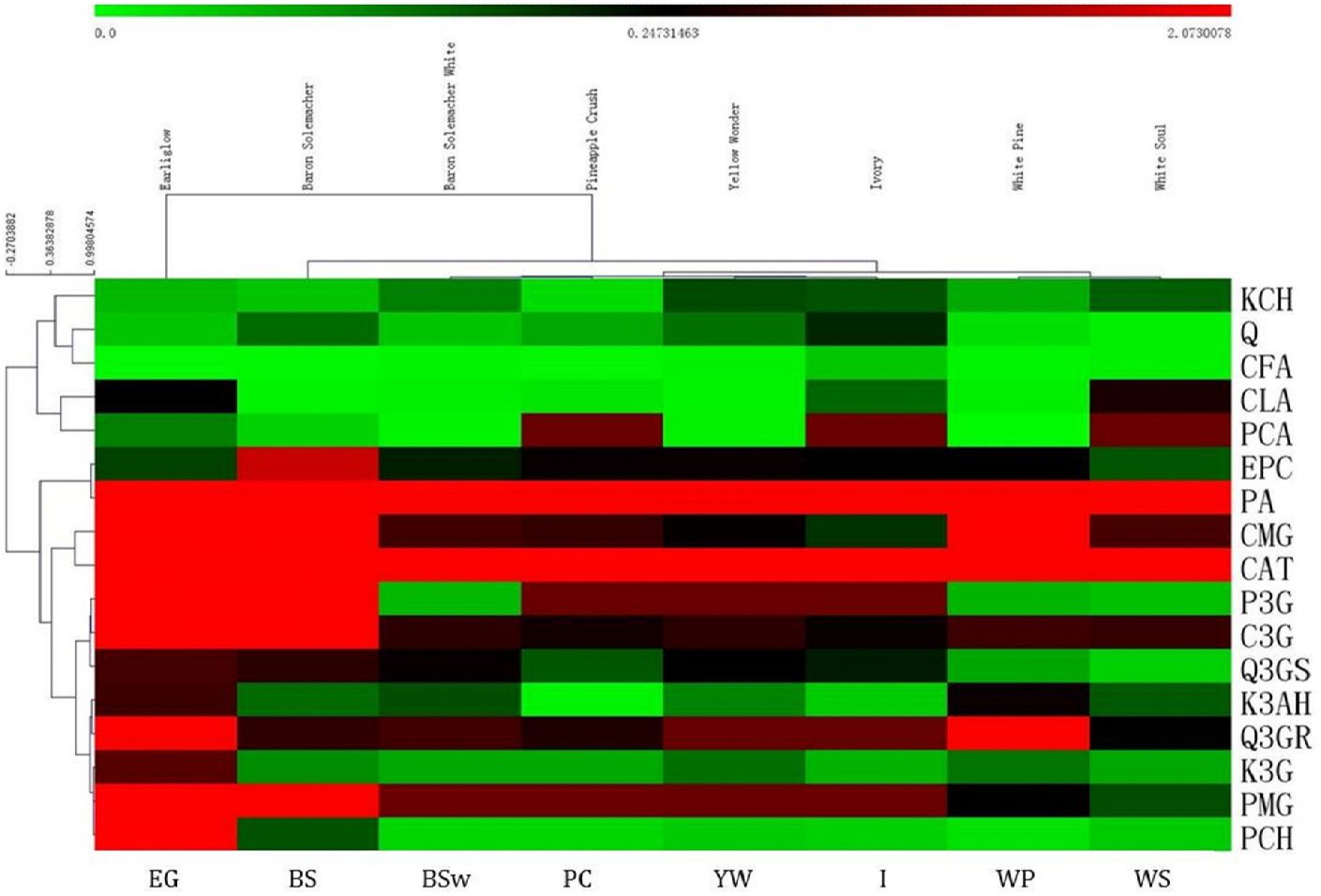



**Figure A.3** Hierarchical cluster analysis (HCA) of *Fragaria* genotypes. *F. vesca* genotypes, BS, red Baron Solemacher (BS), and white Baron Solemacher White (BSW), Ivory (I), Pineapple crush (PC), White Soul (WS), and Yellow Wonder (YW), and the *F.* X *ananassa genotypes* red

Earliglow (EG) and white White Pine (WP) based on phenylpropanoids/flavonoid metabolites. Cell colors reflect the quantity of a metabolite based on the color scale at the top.

3-glucoside and *p*-coumaroyl hexoside, and the latter with a much higher amount of epicatechin. Interestingly, the white forms Baron Solemacher White and Pineapple Crush clustered with the red forms. Also, White Soul (*F. vesca*) and White Pine (*F.* x *ananassa*) clustered separately from the other white genotypes. This may be explained by the higher amount of cyanidin-3-glucoside and quercetin-3-glucuronide for White Pine and higher accumulation of cholorogenic acid in White Soul. Among the white *F. vesca*, Baron Solemacher White and Pineapple Crush were most similar, Yellow Wonder and Ivory were most similar, and White Soul was unique.

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